Saskatchewan

# A D F

## Agriculture Development Fund

# FINAL REPORT

**19960213**

**ENHANCED BIOCIDE EFFICACY AGAINST MEAT PROCESSING PATHOGENS**

**Funded by**
**The Agriculture Development Fund**

**March 2002**
**Prepared by: University of Saskatchewan**

SAF
Saskatchewan Agriculture & Food
Adding value to agriculture...through research and development

102-04943/

# Enhanced biocide efficacy against meat processing pathogens
*and*
# Inhibition of meat processing pathogens using antagonistic microbial communities

Project 96000213
RES.52 BR

**Final Report**

Darren R. Korber
Department of Applied Microbiology and Food Science,
*51 Campus Dr., University of Saskatchewan, S7N 5A8*

***Abstract***

The objective of this project was to enhance the efficacy of trisodium phosphate (TSP) against foodborne bacteria by manipulating the osmotic environment (the PDP process). TSP efficacy using PDP was tested using meat spoilage and pathogenic bacteria in flow cell biofilms and on adipose tissue. PDP enhanced the efficacy of TSP of spoilage and pathogen biofilms grown in flow cells. However, when similar experiments were conducted using pork tissue, PDP failed to significantly enhance TSP efficacy. The exception was *Listeria monocytogenes*, which was sensitive to TSP after PDP. Factors contributing to the failure of PDP to enhance the activity of TSP in meat systems are thought to involve the alkaline pH stress response, as well as the chemically complex buffered nature of meats. The antimicrobial mechanism of TSP against *Salmonella* Enteritidis was further investigated to clarify why some cells survive antimicrobial treatments and others die. Results showed that TSP's high pH and tendency to sequester metal ions causes outer membrane damage, release of intracellular components and cell death. Studies to determine whether the PDP process is suitable for use with other food grade antimicrobial agents, or for non-food biofilm control, are ongoing.

*Executive Summary*

The primary aim of this research project was to evaluate a procedure for the enhancement of the efficacy of foodgrade antimicrobial agents. The efficacy of many biocides are dependent upon their delivery to reach the cell membrane, and in some cases, to enter the cytoplasm. To facilitate this delivery, we developed a two-step process whereby a NaCl pre-treatment (which plasmolyzes cells) is applied before the application of the antimicrobial agent. Plasmolysis only occurs when cells with a functional, semi-permeable membrane are placed in a strong osmotic strength (hypertonic) solution, where cells lose internal water in an attempt to equilibrate. When this strong osmotic pressure is relieved, plasmolyzed cells take up surrounding water, deplasmolyzing, or rehydrating. It is during deplasmolysis that the efficacy of antimicrobial agents may be enhanced if an antimicrobial compound is present in the deplasmolysis solution. This process is hereafter referred to as plasmolysis-deplasmolysis (PDP). During our study, PDP was used in conjunction with TSP (referred to as PDP-TSP) against a number of wild type meat spoilage and pathogenic bacteria. Efficacy of this process was assessed in biofilms grown in flow cells as well as on pork adipose tissue.

A number of antimicrobial agents have been tested during this project (e.g., nisin, myristyl-L-methionine, benzoic acid, hydrogen peroxide), however TSP was found to be the most efficacious compound with current and potential application against red meats. The process plasmolyzed attached bacteria (biofilms) using a 1.5 M NaCl solution, and subsequently deplasmolyzed the same cells using a low osmotic strength solution containing TSP. *Escherichia coli*, *Salmonella* Enteritidis, *Pseudomonas* sp., *Listeria monocytogenes*, and *Brochothrix thermosphacta* strains were grown as pure culture biofilms in glass flow cells, and then treated with either 0.1, 0.25, 0.5, 0.625, or 1.0 % TSP, or the same TSP concentrations delivered in conjunction with PDP. Laser microscopy, a viability probe, and image analysis were then used to determine the abundance of living and dead cells remaining following each treatment. With the exception of *L. monocytogenes* (which was resistant to TSP concentrations up to 5%), PDP enhanced the sensitivity of the test strains to TSP. Of practical relevance was the observation that when similar experiments were conducted with the panel of test bacteria on the surfaces of pork adipose tissue, much higher TSP concentrations (5%) were necessary to result in significant decreases in the number of viable cells. In the meat system, PDP generally failed to enhance TSP efficacy. One interesting exception to this trend was the human pathogen, *L. monocytogenes*, was more sensitive to TSP on pork tissue when pretreated with 1.5 M NaCl. The failure of PDP to enhance the activity of TSP in meat systems likely involves a number of factors, including the innate ability of cells to resist high pH, and more importantly, the fact that meat tissue is quite complex as well as chemically buffered. For this reason, there remains the potential for the PDP process to see application in conjunction with other food grade antimicrobial agents, or in non-food biofilm control applications.

The mode of action of TSP on *Salmonella* Enteritidis cells was determined using a series of comparative studies between different concentrations of TSP, and NaOH solutions adjusted to the same pH values as the TSP solutions. At 2.5 % TSP, or pH 11.0 NaOH, the number of surviving *S. enteritidis* cells decreased by at least 50 % within 5 min. In contrast, no reduction in *S. enteritidis* viability was seen even after 1 h exposure to treatment solutions containing TSP adjusted to pH 7.0. The damage to cell membranes following TSP treatment was indicated by an increased uptake of PI (due to membrane damage) and loss of osmotic responsiveness.

In cells treated with TSP or alkaline pH, reductions in cell viability correlated with loss of membrane integrity, whereas in treatments with different concentrations of TSP (pH 7.0), no loss of cell viability or loss of membrane integrity could be observed. Thus, the major antimicrobial effect of TSP appears to be linked with high pH. Also, TSP treated cells revealed a considerable proportion of DNA and protein became released following TSP or high pH treatment. Similar release of

lipopolysaccharides was observed in filtrates of TSP or alkaline pH treated cells suspensions, compared to control and TSP treatments whose pH has been adjusted to 7.0. Transmission electron microscopy morphological analyses of cell thin sections revealed that TSP or alkaline pH treated cells caused cell lysis by disruption of the cytoplasmic and outer membrane. Overall, these results support the view that the high pH during TSP treatment along with sequestration of metal ions in the outer membrane by TSP, led to membrane damage, release of intracellular components and cell death.

How intestinal pathogens respond to and survive environmental stresses has been the focus of much research, and includes detailed genetic and physiological investigations of the response of global regulatory systems to heat stress, oxidative stress, ultraviolet stress, and aerobic-to-anaerobic shifts. Enteric organisms may become acclimated to stress conditions, rendering surviving pathogens more resistant and often more virulent. This acclimation response is significant for the efficacy of alkaline compounds such as TSP, as sublethal treatment with an agent like TSP can subsequently render the organisms more resistant to other normally-lethal treatments. Thus, this research project sought to not only identify the mode of antimicrobial action of TSP against *Salmonella sp.*, but also to elucidate physiological and molecular responses of *S. enteritidis* acclimated to sublethal concentrations of TSP.

The resistance of stationary phase *Salmonella enteritidis* to lethal challenges, following exposure to sublethal TSP treatment was examined. Stationary phase cells of *S. enteritidis* were exposed to sublethal dose of TSP in trypticase soy broth medium for 60 min at room temperature and 200 rpm. Following exposure to TSP, the cells were examined for resistance to 55°C, 2.5 % TSP, pH 11.0, pH 2.6 and 30 mM $H_2O_2$ in tryptic soy broth. Survivors were enumerated by plating appropriate dilutions on trypticase soy agar, allowing construction of survivor curves and calculation of D-values. Exposure to TSP did increase *S. enteritidis* cell resistance to 55°C, 2.5 % TSP, and pH 11.0, and sensitized cells to acid treatment (pH 2.6). The above results demonstrate that the primary antimicrobial effect of TSP is that of alkaline pH, a treatment that destroys *S. enteritidis* by disrupting the outer membrane and causing leakage of cellular contents. Acclimation of *S. enteritidis* to sublethal TSP concentrations results in an "alkaline stress-like" response, which includes resistance to higher concentrations of TSP and alkaline pH, increased thermotolerance, and sensitivity to acid (Sampathkumar et al., 2000). Chloramphenicol, which inhibits protein synthesis, in part reduced these acquired resistance traits, suggesting that *de novo* synthesis of stress proteins may be involved. These *S. enteritidis* cells subjected to various treatments were also compared using 1- and 2-dimensional polyacrylamide gel electrophoresis (1D/2D-PAGE) analysis of cytoplasmic and membrane proteins, and other indicators of membrane damage and cell viability (see above).

Flahaut and coworkers (1997) showed that mild pH upshifts (from 7.0 to 8.8) induced the well known heat shock proteins DnaK and GroEL. However, Western blot analyses of SDS-PAGE of total cellular proteins from cell exposed to TSP or alkaline pH did not reveal any significant increase in either GroEL or DnaK. However, 2D-PAGE of total cellular proteins of TSP acclimated cells indicated that significant differences in the induction of a number of proteins did occur from control cell preparations. The logical extension of these findings is the identification of any unique proteins produced in significant quantity, and their comparison with other known proteins and functions in the proteomic databases. This element of research cannot be resolved without employing mass spectrometric analyses. An internal proposal is currently under review that is hoped to provide funding for this important set of analyses.

While the primary goals of this research were centered on improving the antimicrobial efficacy of TSP, we also sought to employ natural microbial communities for protecting meat and meat processing surfaces against colonization by pathogens. A significant part of these efforts were invested in the development of efficacious methods for monitoring of survival, growth, and behavior of specific target organisms from within complex microbial systems. Cloning the green fluorescent protein (*gfp*) gene

from the jellyfish *Aqueoria victoria* into recipient target organisms has proven very suitable in this regard, as it provides a conservative non-toxic fluorescent tracer which may be monitored in a number of ways. This utility of this approach has been furthered by the modification of the spectral adsorption and emission properties of green fluorescent protein in variant protein constructs. We thus transformed meat bacterial isolates using electroporation using the following GFP constructs: *pGFP* (a wild-type GFP), *pEGFP* (a red-shifted variant), and *pGFPuv* (a long-wavelength excitable variant). Bacteria transformed by these different *gfp* plasmid constructs varied in terms of their response to different wavelengths of light, as well as the wavelength of light emission. For example, the *pGFPuv* was optimized for maximal fluorescence when excited by UV light (360-400 nm) as well as for enhanced expression of the *gfp* gene by recipient bacteria. The *GFPuv* variant is consequently well-suited for *gfp* expression and subsequent detection using a hand-held UV light. The *pEGFP* red-shifted variant also contain mutations in the chromophore which shifts the maximal excitation peak to ~490 nm (the major line of excitation for argon ion lasers), resulting in more efficient chromophore excitation. The *pEGFP* fluorescence emission is ~35 fold more fluorescent than wild-type GFP, consequently bacteria expressing these genes are much easier to detect using confocal scanning laser microscopy. We have subsequently demonstrated the utility of gfp-labeled organisms in complex biofilm communities, and have also established appropriate procedures and conditions for ensuring that optimal fluorescence are maintained. Of particular importance is the fact that gfp fluorescence is oxygen dependent, and in slowly growing biofilms, or in oxygen limited environments, the fluorescent signal is often quite weak. The bacteria containing the *gfp* gene must therefore be resuscitated, metabolically speaking, by providing a brief pulse (~1 hr) of rich nutrient under flowing conditions. This serves to permit the new synthesis of gfp protein, as well as enrich the system in oxygen, which diffuses to the bacterial cells. As a consequence, cells containing the *gfp* gene may then be readily detected from within a complex mixture of bacteria, without leading to significant growth (a 1 hr pulse of nutrient under flowing conditions was demonstrated to not cause significant growth). Green fluorescent protein-based approaches have been used to quantify the number of pathogen bacteria residing in complex biofilm communities, as well as determining the number of gfp-labeled target bacteria surviving on meat coupons over time.

*Technical Report*

***Study area 1.*** Efficacy enhancement of trisodium phosphate against spoilage and pathogenic bacteria in model biofilms and on adipose tissue.

***Background***

During the slaughter, dressing and processing of meat animals, spoilage bacteria and pathogens contaminate equipment and exposed meat surfaces (Gill, 1998). Subsequent bacterial attachment (Dickson, 1991) and biofilm formation (Zottola and Sasahara, 1994) is a concern to the industry. Thus, biofilms have been quantified in meat processing plants (Hood and Zottola, 1997) and visualized on meat tissue surfaces (Delaquis et al., 1992).

From a meat processing perspective, the most critical biofilm-related issues include the increased resistance of biofilm bacteria to sanitizers when compared with planktonic cells (Desai *et al.*, 1998; Norwood and Gilmour, 2000; Ronner and Wong, 1993; USDA, 1996) and the difficulty in removing biofilm bacteria from food contact surfaces (Gibson *et al.*, 1999). Because of their ubiquity in processing plants, biofilms have the potential to constitute a long term recontamination threat for meat and meat products (Arnold and Silvers, 2000; Michiels *et al.*, 1997; Mittelman, 1998; Wong, 1998). An understanding of bacterial attachment, as well as biofilm formation and persistence, is therefore fundamental for the development of antibacterial intervention strategies suitable for the meat industry (Cabedo *et al.*, 1997).

Regulatory authorities (USDA 1996) have advocated the use of certain antimicrobials, including trisodium phosphate (TSP), to assist the meat processing sector in meeting pathogen reduction performance standards for red meats. TSP has been approved for use in hog scalding and poultry processing (Morris *et al.*, 1997), and as a spray to be applied to beef carcasses prior to chilling (Dorsa *et al.*, 1997). Research has shown that TSP can facilitate significant reductions in meatborne spoilage bacteria (Dorsa *et al.*, 1998) and pathogens (Cutter and Rivera-Betancourt, 2000; Dickson *et al.*, 1994). Also of importance, TSP was found to be effective in removing attached *E. coli* O157:H7 and *Salmonella* from beef (Kim and Slavik, 1994).

Problems associated with the use of TSP include the relatively high concentrations (8-12%) necessary to obtain measurable reductions in bacterial populations on meat, as well as the associated potential to produce undesirable organoleptic changes (Carneiro de Melo *et al.*, 1998). Approaches which reduce the concentration of TSP required to effect significant reductions in viable cell numbers would have the potential for commercial application. While the mode of TSP action at the level of the bacterial membrane is not completely clear, it appears that high pH plays a large role in it's antimicrobial activity (Carneiro de Melo *et al.*, 1998; Sampathkumar *et al.*, unpublished data). Other possible consequences of TSP application include metal ion sequestration and cell detachment caused by surfactant effects. Since TSP's primary active site appears to be the cell membrane, a number of researchers have used approaches where other membrane active agents are applied in concert with TSP. Thus, TSP has been used in combination with nisin, sodium chlorite-based oxyhalogen disinfectants, lysozyme and chelators in an effort to enhance antimicrobial efficacy (Carneiro de Melo *et al.*, 1998; Cutter and Siragusa, 1995; Mullerat *et al.*, 1995).

The efficacy of many biocides requires their delivery to either the cell cytoplasm or cytoplasmic membrane. To enhance this delivery process, we have developed an approach that involves a NaCl pre-treatment, to plasmolyze cells, prior to the application of the antimicrobial agent. Plasmolysis has previously been used as a physical indicator of viability (Korber *et al.*, 1996), since plasmolysis only occurs when cells which possess a functional, semi-permeable membrane are placed in a hyper-

tonic solution. Under these conditions, cells lose intracellular water while "attempting" to equilibrate with the high osmotic strength extracellular solution. When the osmotic pressure is relieved, plasmolyzed cells take up surrounding water, deplasmolyzing, or becoming rehydrated. It is during the deplasmolysis phase that the efficacy of antimicrobial agents may be enhanced (via facilitated uptake) if these antimicrobial compounds are present in the low osmotic strength deplasmolysis solution. During the present study, plasmolysis-deplasmolysis (PDP) was used in conjunction with TSP against a number of wild type meat spoilage and pathogenic bacteria. Efficacy was assessed in model biofilms grown in flow cells and on pork adipose tissue.

***Project objective***

The objective of these studies was to use the process known as plasmolysis-deplasmolysis (PDP) to enhance the inhibitory activity of trisodium phosphate against foodborne pathogen and spoilage bacteria. These studies were conducted in flow cells, which provided an effective method for screening different concentrations of different antimicrobial agents against a panel of test organisms, and on pork tissue.

***Results***

**The effect of PDP-TSP on biofilm bacteria cultivated in flow cells.** Some organisms used in this study did not adhere well to surfaces and hence a number of isolates were evaluated for their ability to adhere to surfaces and form biofilms. For example, significant variability in terms of adherence and biofilm formation was observed for the 9 pseudomonad and 10 *Brochothrix thermosphacta* meat and processing plant isolates screened (data not shown). Qualitative assessments, performed directly using high magnification microscopy, were performed after 24 h growth on flow cell surfaces and used to identify *Pseudomonas* C531 and *Brochothrix thermosphacta* S521 as isolates most amenable to this study. The remaining organisms used during these experiments (*L. monocytogenes* 4, *E. coli* 8, *S.* Enteritidis) readily attached to flow cell surfaces and formed biofilms.

Biofilms of *Pseudomonas* C531, *B. thermosphacta* S521, *E. coli* 8, *S.* Enteritidis, and *L. monocytogenes* 4 were cultivated in flow cells for 24 h before treatment with either TSP (concentration range 0.125 – 1.0%) or PDP-TSP (at the same concentations of TSP). During these trials it was found that a 1 min treatment with TSP at any of the concentrations tested was insufficient to cause complete killing of *E. coli* 8 or *S.* Enteritidis (Figure 1 A and C, respectively). The two lowest concentrations of TSP (0.125 and 0. 2%) had little impact on the number of living *S.* Enteritidis or *E. coli* cells remaining in the flow cells, with ≥ 95% of the biofilm biomass remaining viable. Following treatment with 0.5 to 1.0% TSP, the relative abundance of viable *S.* Enteritidis and *E. coli* cells remaining deceased in a TSP concentration-dependent manner, although ≥ 65% of each cell population still remained viable. Applying NaCl in conjunction with TSP (PDP-TSP) enhanced TSP efficacy; PDP-0.5% TSP resulted in ≥ 95% killing following application to either *S.* Enteritidis or *E. coli 8*, with higher TSP concentrations (0.65 and 1.0%) effectively eliminating all CLSM-detectable bacteria.

Treatment of *Pseudomonas* C531 biofilms with PDP-TSP also led to enhancement of TSP efficacy (Figure 1 D). Application of 0.2% TSP caused ≥ 95% killing of *Pseudomonas* C531 biofilm cells while PDP-0.1% TSP resulted in complete killing of all cells. *B. thermosphacta* S521 (Figure 1 B) also showed enhanced TSP susceptibility when applied in conjunction with PDP, however the difference between TSP and PDP-TSP treatments (at the same TSP concentration) was less pronounced than for either *E. coli, S.* Entertidis, or *Pseudomonas*. *L. monocytogenes* 4 biofilms were not sensitive to TSP with or without the PDP process, even at 10% TSP.

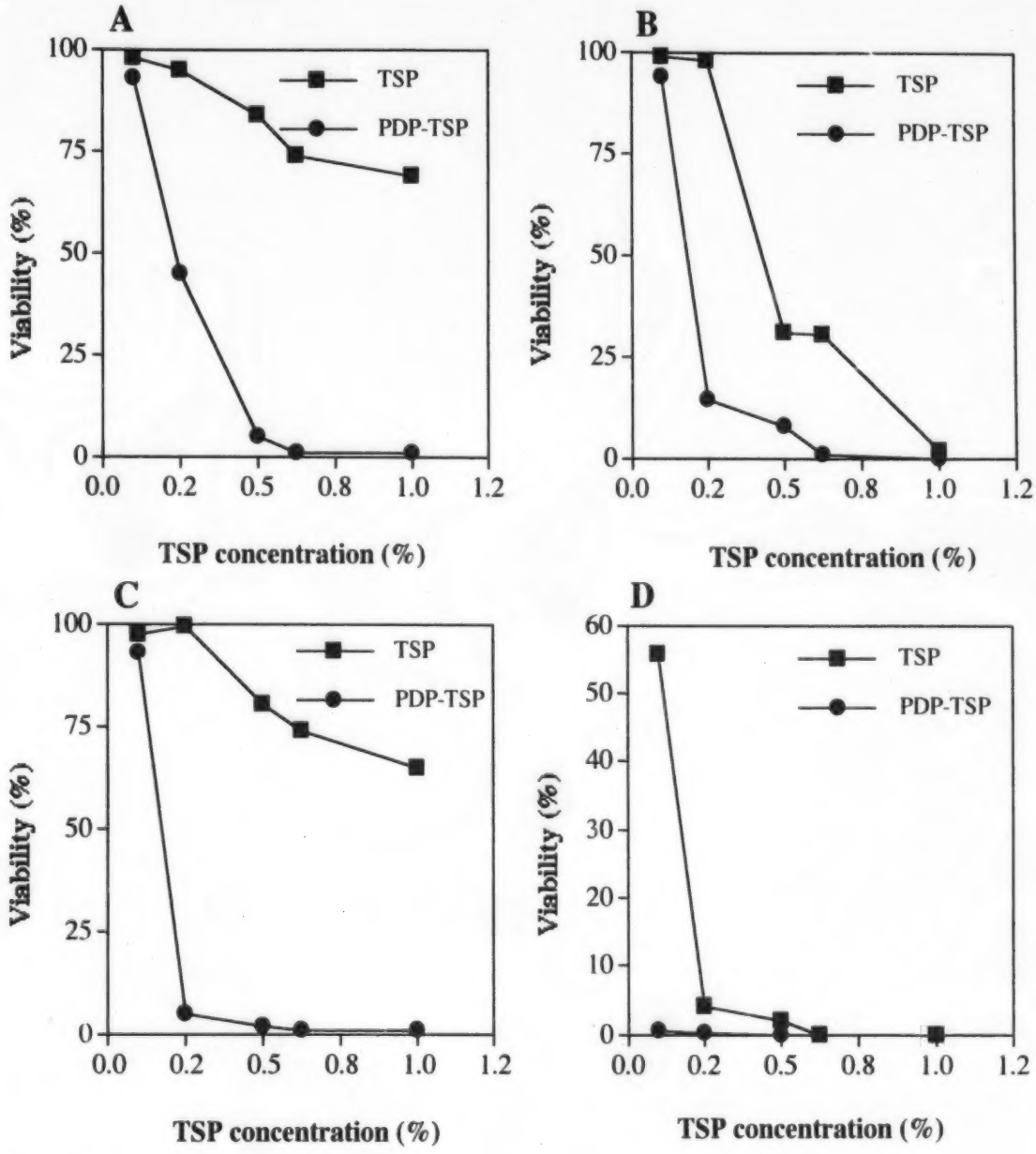



**Figure 1 A-D.** The effect of PDP on the viability of biofilm bacteria grown in flow cells for 24 h following various TSP or PDP-TSP treatments. Viability of biofilm bacteria was determined using the *Bac*Light viability probe, CLSM, and image analysis. **A, B, C,** and **D** show the percent viable cells remaining for *E. coli* 8, *B. thermosphacta* S521, *S.* Enteritidis, *Pseudomonas* strain C531, respectively.

While the combination of time, flow rate and nutrients were chosen to result in the development of relatively dense biofilms, colonization patterns and 24 h biofilm cell densities varied in a species-dependent manner (Figure 2A-D). The patterns of viability probe staining at the biofilm base (0 $\mu$m section depth) following application of sublethal TSP concentrations to *E. coli, B. thermosphacta, S.* Entertidis and *Pseudomonas* biofilms are shown in Figure 3A-D. Notably, *E. coli* was the only

strain where dense accumulations of cells appeared to impact the survival of embedded bacteria. Regions of surviving *E. coli* 8 cells were typically observed in areas of high cell density (in the xy plane) and covered by up to 10 $\mu$m of overlying biofilm biomass.

**The effect of PDP-TSP on bacteria on adipose tissue.** The data in Figure 4 show the effects of NaCl and TSP upon the growth of *Pseudomonas* C531 and *L. monocytogenes* 4 on pork adipose tissue discs. In comparison to water-treated control tissue, NaCl had only marginal effects on bacterial numbers during the 8 day storage period. In contrast, TSP produced an immediate 1 log reduction in pseudomonads (Figure 4A) and *Listeria monocytogenes* (Figure 4B). Consequently, bacteria recovered from TSP-treated tissue generally remained about 1 log lower than controls during 8 days storage, but the rates of bacterial growth were similar.

When *Pseudomonas*-inoculated tissue discs were pretreated with 1.5 M NaCl immediately prior to treatment with 5% TSP (Figure 4A), there was only slight enhancement of antimicrobial activity after 4 days storage. At this time there was a 2.17 log reduction in pseudomonads compared to a 1.53 log reduction with TSP, alone. Similar trends were observed in an identical trial with another wild type strain, *Pseudomonas* D23 (data not shown).

*L. monocytogenes* 4 appeared more susceptible to the synergistic effects of PDP-TSP (Figure 4B). After 2 and 4 days post-treatment there was 1.96 and 1.81 log reduction in *L. monocytogenes*, respectively, when PDP was used in conjunction with 5% TSP. This was somewhat greater than the 1.19 and 0.64 log reduction observed at the same sampling times for tissue treated with TSP alone. Attempts to enhance this synergistic effect using higher concentrations of TSP (10%) were not successful (data not shown).

Growth on adipose tissue discs was also compared with the 3 other bacteria (*B. thermosphacta* S521, *S.* Entertidis, and *E. coli* 8, using an identical experimental design. Antimicrobial efficacy was greatest at 2 to 4 days post-treatment and, for convenience, treatment comparisons were arbitrarily summarized after 4 days of storage at 4°C. Data in Table 1 compare the reduction in log bacterial numbers (water control minus antimicrobial treatment) for all 5 bacteria, 4 days post-treatment with NaCl or TSP alone, or applied in sequential treatments (PDP-TSP).

The data showed that NaCl by itself produced a limited reduction in bacterial numbers (0.05 to 0.78 log cycles). The Gram positive bacteria seemed more resistant to TSP, with log reductions of 0.84 and 0.64 for *B. thermosphacta* and *L. monocytogenes*, respectively. When the Gram negative species were similarly treated with 5% TSP, reductions of 1.53, 1.59 and 2.88 log were respectively observed for pseudomonads, *E. coli* and *S.* Enteritidis.

When inoculated tissues were pre-treated with 1.5 M NaCl immediately prior to the 5% TSP treatment, there was only limited evidence of enhanced bacterial activity over and above that recorded with TSP alone. With *B. thermosphacta* and *S.* Enteritidis there was no enhancement of susceptibility to TSP. When *Pseudomonas* C531 and *E. coli* 8 were the test strains, the combined PDP-TSP treatment produced only a marginal increase in bacterial susceptibility (~0.5 log) in comparison to the antibacterial effects of TSP alone. As described for Figure 4B, the only pronounced improvement of TSP efficacy was observed with *L. monocytogenes* 4, where sequential treatment with NaCl and TSP produced more than a 1 log decrease in bacterial populations over and above that found with TSP alone.

Similar results were observed for *E. coli* and *S.* Enteritidis inoculated adipose tissue stored at 10°C, where bacterial proliferation was significant (data not shown). Results with *B. thermosphacta* S521 were also confirmed in an identical trial using a different meat isolate, *B. thermosphacta* B2 (data not shown).

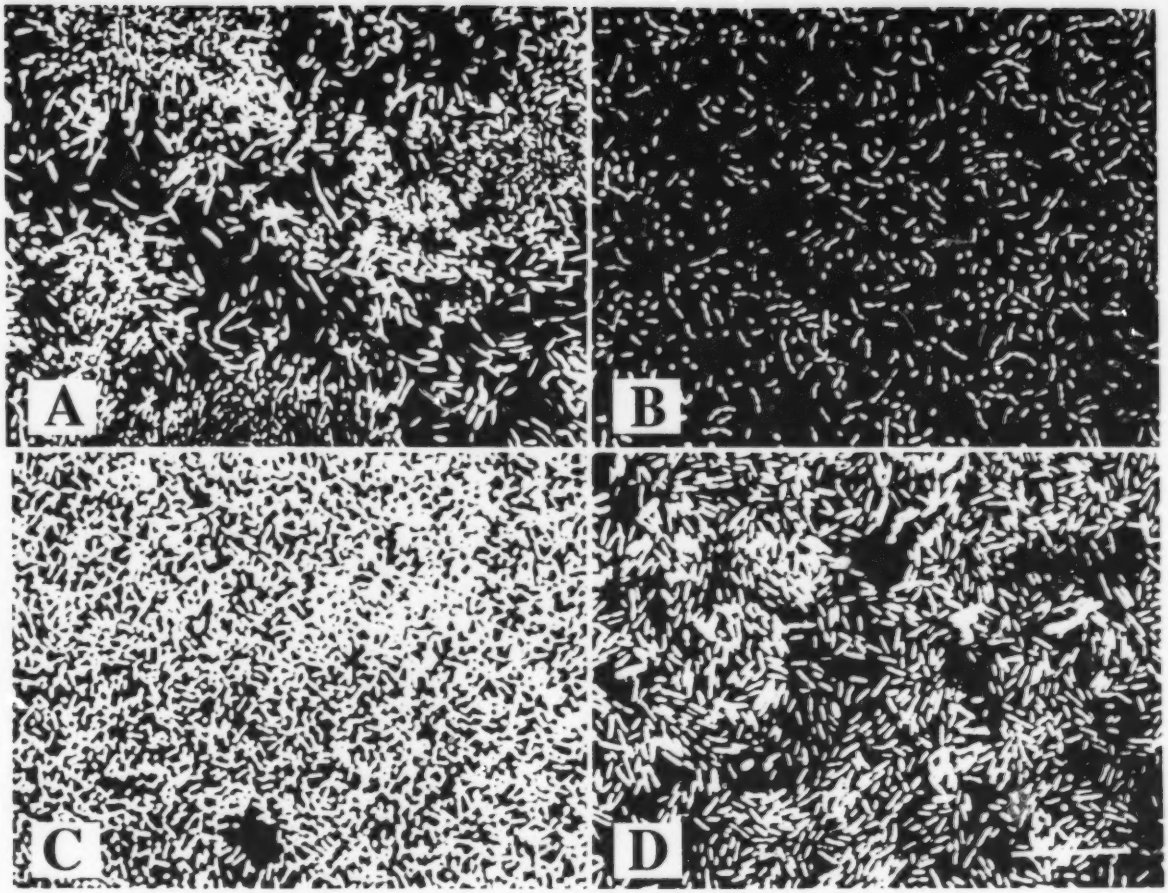



**Figure 2.** Representative CLSM thin sections (0 $\mu$m section depth) of 24 h biofilms of *E. coli* 8 (A), *B. thermosphacta* S521 (B), *S.* Enteritidis (C), and *Pseudomonas* C531 (D) positively-stained with the green fluorescing, membrane permeant nucleic acid stain, SYTO 9. Bar = 25 $\mu$m.

### *Discussion*

The objective of this study was to determine whether plasmolysis-deplasmolysis (PDP) could be used to enhance the killing efficacy of TSP against attached bacteria of concern to the meat processing industry. The first stage of the PDP process plasmolyzes the biofilm bacteria by addition of a high osmotic strength (1.5 M NaCl) solution. After a 1 - 3 min period, the osmotic stress is relieved by pumping a low osmotic strength solution supplemented with trisodium phosphate. It was hypothesized that PDP could be used to facilitate the delivery of the antimicrobial agent to the cell and across the plasma membrane. This premise was first examined *in situ* against biofilms cultivated in a flow cell model system using CLSM and a fluorescent viability probe (Korber *et al.*, 1997). The PDP process was then tested against the same organisms on adipose meat discs treated in a similar fashion.

While there has been an increase in the number of *in vitro* studies applying fluorescent indicators of cellular viability to bacteria following various treatments, relatively few of these studies have utilized direct, fluorescent methods for quantifying the efficacy and antimicrobial agents against biofilm bacteria (Auty *et al.*, 2000; Korber *et al.*, 1994, 1997). During the present study, flow cells and digital imaging were used to provide initial evidence that the efficacy of TSP against biofilm bacteria could be enhanced by manipulating the osmotic strength of the system prior to TSP application.

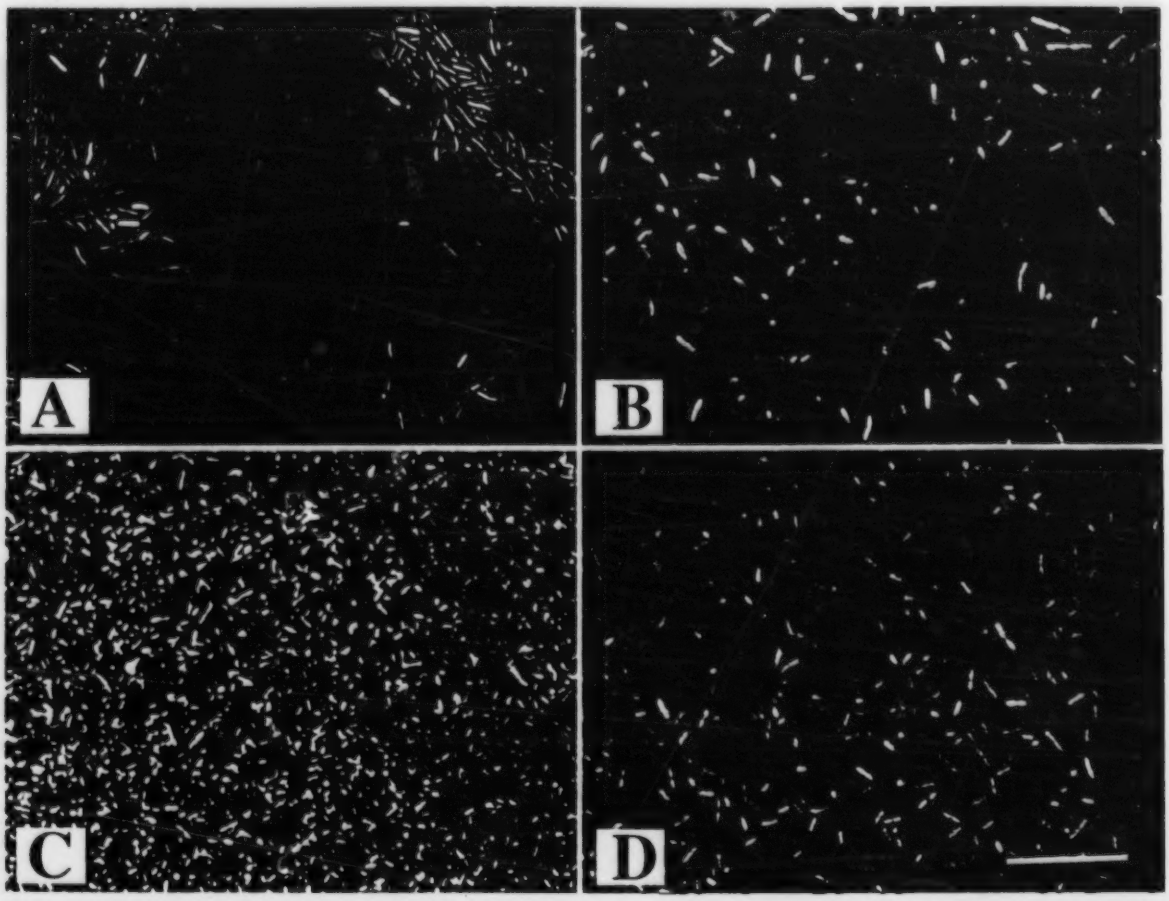



**Figure 3.** Representative dual-channel CLSM optical thin sections (0 $\mu$m section depth) of 24 h biofilms of *E. coli* 8 (A), *B. thermosphacta* S521 (B), *S.* Enteritidis (C), and *Pseudomonas* C531 (D) following sublethal treatment with TSP and staining with the *Bac*Light viability probe. The red-green pseudocolor presentation shows the result of merging fluorescent signals from the green and red wavelengths (corresponding with living and dead bacteria, respectively). Bar = 25 $\mu$m.

TSP applied alone exhibited antimicrobial activity against all biofilm bacteria examined during this study, with the exception of *L. monocytogenes* 4. The Gram negative isolate, *Pseudomonas* C531, was most susceptible to the action of TSP when examined in flow cells, with 0.125% TSP causing a 45% reduction of cell viability. When PDP-0.125% TSP was applied against this organism, < 0.5% of the biofilm bacteria remained viable. The two other Gram negative organisms tested (*E. coli* 8 and *S.* Enteritidis) were also sensitive to TSP, however even the highest TSP concentration tested (1.0%) failed to result in more than a 35% reduction in living cells. During an earlier study, Korber *et al.* (1997) demonstrated that 15 s exposure to 10% TSP was sufficient to completely kill all *S.* Enteritidis biofilm bacteria, however incomplete killing resulted when the same biofilms were located between artificial crevices (1,500 $\mu$m wide by 500 $\mu$m high). During the present study, only when PDP was applied in conjunction with ≥ 0.5% TSP was enhancement of TSP killing increased to ≥ 95%. For the Gram negative organisms, the maximum efficacy enhancement (reduction in viability) for each strain was 95, 69, and 55% for *S.* Enteritidis, *E. coli* and *Pseudomonas*, respectively. It was hypothesized that the PDP approach would result in a similar, or greater, enhancement of TSP efficacy when applied in a pork tissue assay system employing a 5-fold higher TSP concentration (5%).

To determine whether PDP efficacy enhancement of TSP in flow cells would function in the meat environment, pork adipose tissue discs were artificially inoculated with meat spoilage bacteria

**Table 1.** Effect of 1.5 M NaCl and 5% trisodium phosphate on the recovery of bacteria from inoculated pork adipose tissue

| Bacteria | Reduction in Bacterial Numbers (log cfu/cm$^2$)[1] NaCl | TSP | NaCl + TSP | SE |
|---|---|---|---|---|
| *Pseudomonas* C531 | 0.78 | 1.53 | 2.17 | 0.09 |
| *B. thermosphacta* S521 | 0.05 | 0.84 | 0.30 | 0.19 |
| *L. monocytogenes* 4 | 0.30 | 0.64 | 1.81 | 0.25 |
| *E. coli* 8 | 0.07 | 1.59 | 2.11 | 0.24 |
| *S. enteritidis* ATCC 4931 | 0.46 | 2.88 | 2.78 | 0.18 |

[1]Values are the difference between the control treatment (water only) and the biocide treatment after 4 days at 4°C. Data are Least Squares Means of bacterial counts of 5 adipose tissue discs. Initial level of inoculated bacteria was log 5 to 6 cfu/cm$^2$.

(*Pseudomonas*, *B. thermosphacta*) and pathogens (*L. monocytogenes*, *E. coli*, *S.* Enteritidis). In accordance with the findings of other researchers, relatively high concentrations of TSP used alone could be expected to produce a 1 to 2 log reduction in bacteria attached to beef tissue (Dorsa *et al.*, 1997; Kim and Slavik, 1994). In contrast, the more recent results of Cutter and Rivera-Betancourt (2000) indicated that pathogens associated with beef surfaces could be substantially reduced by more than 3 log cycles using 10% TSP spray treatments.

In the current study, TSP was utilized at a concentration of 5% to produce a measurable, but not maximum, reduction in bacterial numbers (determined during preliminary studies, data not shown). A range of TSP concentrations (4 to 12%) were previously evaluated by others (Morris et al., 1997) to investigate possible approaches to treat commercial pork carcasses. It was concluded that a concentration of 8% TSP or greater would achieve about a 2 log reduction of *Salmonella* on pork skin during contact times of 5 to 15 s. Under the same conditions, the total aerobic plate count was unaffected.

Osmotic stress caused by NaCl has been found to improve the efficacy of acetic acid in reducing *L. monocytogenes* and *S. typhimurium* numbers on beef tissues (Dickson, 1990). There is also evidence that a combination of NaCl and polyphosphates can act synergistically to limit the growth of *Aeromonas hydrophila* in ground pork (Palumbo *et al.*, 1995) and *Listeria monocytogenes* in milk (Marriot *et al.*, 1996). However, these treatments involved the simultaneous application of NaCl and antimicrobial agent, whereas the PDP process is sequential, with NaCl pretreatment followed by exposure to TSP.

Of the 5 bacteria evaluated during the present study, *L. monocytogenes* was the only organism demonstrating a marked increase in sensitivity to TSP following NaCl pre-treatment of inoculated pork adipose tissue. This was of interest since *L. monocytogenes* was most resistant to PDP-TSP treatment in the model biofilms. Other researchers have similarly found that biofilm cells of *L. monocytogenes* were the most resistant of a number of foodborne bacteria to TSP (Marriot *et al.*, 1996; Somers *et al.*, 1994). Somers *et al.* (1994) reported that *L. monocytogenes* was highly resistant to TSP treatment; a 12% TSP treatment for 5 min or a 8% TSP treatment for 10 min was required to obtain a 1 log reduction in *Listeria* cell numbers. During the present study, TSP concentrations in the range of 0.1 to 1.0% failed to inhibit *L. monocytogenes* 4 biofilms cultivated in flow cells whether applied alone

in conjunction with PDP (data not shown). Concentrations of 2.0, 5.0 and 10% were subsequently tested, with and without the PDP process, without significant effect. A recently published study concluded that one must be cautious in selecting a concentration of TSP for use in foods since low concentrations may actually enhance the growth of *L. monocytogenes* (Capita *et al.*, 2001). It has been well-documented for various pathogenic organisms that the application of sub-lethal concentrations of various stress factors (e.g., heat, acid, osmotic shock) results in the acquired resistance to otherwise lethal stress factors (Berry and Cutter, 2000; Lou and Yousef, 1997), hence possible collateral effects stemming from TSP application require further investigation.

There are additional reports indicating that antimicrobials which are highly efficacious in broth systems often loose activity in the more complex environments of foods (Greer *et al.*, 2000). It was recently shown that TSP was far less active in a cooked, ground meat medium in comparison to commercial broth systems (Wolfaardt *et al.*, 1994). The presence of proteins (i.e., bovine serum albumin) and complex organic molecules have been shown to impair the efficacy of antimicrobial agents (Mullerat *et al.*, 1995). Also, fresh red meat is a highly buffered system and since the mode of TSP action is related to high pH, long term effectiveness may be impaired.

Concerns for undesirable organoleptic changes associated with the use of high-pH TSP may prohibit commercial application at the concentrations (8-12%) required to reduce bacterial numbers on red meat (Carneiro de Melo *et al.*, 1998). However, there are no data to support this contention (Morris *et al.*, 1997). It is unfortunate that the plasmolysis-deplasmolysis approach appears ineffective in meat systems, since the PDP-TSP synergy was very pronounced in the biofilm model in flow cells and hence may have provided an acceptable means of enhancing TSP efficacy and reducing the amount of antimicrobial needed to be added to meat.

There is considerable value in the use of *in vitro* model systems such as flow cells for efficacy testing antimicrobial compounds against foodborne organisms of concern. The natural habitat of organisms associated with meats and meat processing are solid liquid interfaces (e.g., meats, plastics, stainless steel) (Bagge *et al.*, 2001; Kumar and Anand, 1998; Michiels *et al.*, 1997), hence this approach is significant when considering reports of increased antimicrobial resistance of biofilm bacteria when compared with their planktonic counterparts (Anderl *et al.*, 2000; Brown *et al.*, 1988; Desai *et al.*, 1998; Kumar and Anand, 1998; Norwood and Gilmour, 2000; Ronner and Wong, 1993; Stewart *et al.*, 1998). Also, the composition of the overflowing medium can rapidly be changed so as to permit the pulse application of various test and wash solutions (Korber *et al.*, 1996, 1997), precluding procedural difficulties which might exist in testing these treatments on meat surfaces. Lastly, the growth, viability, and abundance of biofilm bacteria can be examined *in situ* using fluorescent probes (Auty *et al.*, 2000; Lawrence *et al.*, 1997; Mattick *et al.*, 2000), often providing other indications of antimicrobial effects or other correlative relationships that may not be observed using growth-based methods of monitoring antimicrobial efficacy. For example, the tendency for bacteria positioned at the base of *E. coli* biofilms to survive TSP treatment suggests that either these cells were intrinsically more resistant to TSP than were other biofilm bacteria, or that the overlying bacteria and their exopolymeric substances somehow prevented TSP from exerting antimicrobial effects. Overlying biofilm material (cells and EPS) has commonly been contended to provide a protective mechanism. This, in part, may contribute to the observed differences in antimicrobial resistance between biofilm bacteria and their planktonic counterparts (Brown *et al.*, 1988).

In conclusion, food and food processing equipment, together with the highly buffered system typical of meats, offer microorganisms a complex environment that suppress the efficacy of antimicrobial agents. Additional study is required to determine whether potential exists for integrating the PDP strategy with a different antimicrobial agent, or whether PDP can more effectively be integrated with other industrial sanitization situations.

*Study 2.* Trisodium phosphate permeabilizes *Salmonella enteritidis* by disrupting the outer membrane.

**Background**

*Salmonella* species continues to be commonly associated with cases of foodborne disease in developed countries. In the United States in 1999, incidence per 100,000 populations was highest for salmonellosis (17.7), followed by campylobacteriosis (14.8), and shigellosis (4.0) (CDC, 2000). Enteric pathogens usually contaminate the surface of raw animal products during slaughter and primary processing (scalding, defeathering or dehiding, rinsing, cutting, mixing, and grinding, etc.) and can cause disease if these products are undercooked (CDC, 1993).

A wide array of methods for reducing the load of potential pathogens on the surfaces of meat products has been developed. For example, ionizing radiation (14), organic acid sprays (Delmore *et al.*, 2000; Dickson, and Anderson, 1992; Podolak *et al.*, 1996; Smulders and Greer, 1998.), and polyphosphate (e.g., trisodium phosphate) dips/sprays (Delmore *et al.*, 2000) have all been used to reduce the numbers of bacterial pathogens present on raw animal products following processing. Trisodium phosphate is generally recognized as safe (GRAS) by the Food and Drug Administration and has been approved by the United States Department of Agriculture for use as a food ingredient (Federal Register, 1982) and to reduce *Salmonella* contamination during poultry processing (Federal Register, 1994). At 8 to 15 % w/v, TSP has been demonstrated to kill gram-negative pathogens artificially inoculated on surfaces of foodstuffs (Dickson *et al.*, 1994; Dorsa *et al.*, 1997; Hwang and Beuchat, 1995; Zhang and Beuchat, 1996; Xiong *et al.*, 1998ab; Taormina and Beuchat, 1999). Log reductions in viable counts of 1.4 and 0.9 were obtained for *Escherichia coli* O157:H7 and *Salmonella typhimurium*, respectively, on beef adipose tissue, though significantly lower reductions (0.9 and 0.5, respectively) were observed on fascia (Kim and Slavik, 1994a). On chicken carcasses, Slavik *et al.* (1994) and Kim *et al.* (1994) showed that TSP treatment at 10 or 50°C reduced *Salmonella* counts by 1.6 to 1.8 log cycles per carcass, and in a scanning electron microscopic study, Kim and Slavik (1994b) showed TSP effectively removed inoculated *Salmonella* from chicken skin. The process involves immersing post-chill whole birds for 15 min in a 10 % solution of Av-Gard™ TSP, allowing the excess TSP solution to drip from the bird, presumably leaving minimal amounts of phosphate on the carcass (Rhône-Poulenc, 1992). Taste tests show that TSP treatment at these relatively high concentrations have no effect on flavour, texture or appearance of treated poultry (Capita *et al.*, 2000; Hollender *et al.*, 1993; Kim and Marshall, 1999), however there is some disagreement on this point.

Very little is known of the actual mechanisms of TSP antimicrobial activity. To date, work has mainly focussed on evaluating the efficiency of *Salmonella* removal by the process (Coppen *et al.*, 1998; Delmore *et al.*, 2000; Xiong *et al.*, 1998ab, Yang *et al.*, 1998). Bender and Brotsky (Bender and Brotsky, 1992) speculated that TSP functions as an antimicrobial by removing a fat coating on poultry skin, thereby allowing bacteria to be washed from the surface of the bird more effectively (Giesse, 1992). However, there is also a clear body of evidence pointing to membrane damage caused by the high pH of TSP solutions as the primary cause of bacteriocidal activity (Mendonca *et al.*, 1994). Other possible modes of TSP action include sequestration of metal ions (Lee *et al.*, 1994) and enhancing detachment of bacteria from food surfaces (Kim and Slavik, 1994a).

During the present study, the role of high pH during TSP treatment of *Salmonella enterica* serovar Enteritidis (ATCC 4931) was delineated using a series of comparative studies involving different concentrations of TSP, and NaOH solutions adjusted to the same pH. Direct and indirect indicators of cell survival, membrane damage, and cellular leakage were employed to determine the relative contributions of possible antimicrobial effects.

**Project objective.**

The objective of these studies was to determine the mode of antimicrobial activity of trisodium phosphate against the foodborne pathogen *S.* Enteritidis. Membrane effects and expression of cytoplasmic and outer membrane proteins detailed how sub-lethal treatment of *S.* Enteritidis cells with TSP impacted the survival of cells subsequently challenged with alkaline antimicrobial agents.

**Results**

***Effect of TSP on cell viability***

The viability of *S.* Enteritidis exposed to TSP decreased as a function of time. D values (Table 1) show the D-values for acclimated or unacclimated (control) *S.* Enteritidis cells exposed to either 2.5% TSP, pH 11, 55°C, pH 2.6, or 30 mM $H_2O_2$. It can be seen from the D-value data that significant ($P< 0.01$) cross-protection from sublethal TSP treatment was provided to cells subsequently treated with heat, high pH solutions and 2.5% TSP. Chloramphenicol treatment resulted in a reduction in the protective effect observed following pretreatment with 1.5% TSP, suggesting that gene expression and subsequent de novo protein synthesis play a role in the acquired stress resistance phenomenon.

Numbers of *S. enteritidis* decreased faster when exposed to increased concentrations of TSP (Figure 1A). A similar reduction in number of surviving *S.* Enteritidis was observed when cell suspensions were exposed to pH 10.0, 10.5 or 11.0 (pH of treatment solutions containing 1.5, 2.0 and 2.5 % TSP respectively) made with NaOH (Figure 1B). Stationary phase cells of *S.* Enteritidis were more resistant to 1.5 % TSP, or pH 10.0, as they showed only a 2-log reduction in numbers after a 1 h exposure (Fig 1A&B). However, no reductions in cell numbers were observed when the cells were exposed to different concentrations of TSP where the pH had been adjusted to 7.0 (Figure 1C).

**Table 1.** D-values of *S. enteritidis* ATCC 4931.

| | D-value (min)[a] | | | | |
|---|---|---|---|---|---|
| **Treatment** | **55°C** | **2.5 % TSP** | **pH 11.0** | **pH 2.6** | **30 mM $H_2O_2$** |
| **Control** | 2.53A[b] | 1.37A | 0.85A | 1.65A | 4.12A |
| **1.5 % TSP** | 6.46B | 2.86B | 1.75B | 1.29B | 3.70A |
| **1.5 % TSP + Chloramphenicol** | 3.98C | 2.43C | 1.66B | 0.57C | 2.95B |

[a] D-values are average from three separate experiments.
[b] Values in the same column followed by different letters are significantly different ($P< 0.01$).

***Propidium Iodide uptake***

Cells exposed to TSP or high pH solutions (NaOH) showed a concentration dependent increased in PI uptake (Figure 2). However, when cells were exposed to treatment solutions containing different concentrations of TSP where the pH had been adjusted to 7.0, the PI uptake by cells was similar to that of the control (Figure 2).

To understand the effect of TSP or alkaline pH on the outer membrane of *S.* Enteritidis , stationary phase cells were pre-treated with EDTA (removes stabilizing divalent cations from the outer membrane by chealtion). EDTA pre-treatment of *S. enteritidis* significantly enhanced PI uptake following TSP or alkaline pH exposure, compared to TSP or alkaline pH exposure without EDTA pre-treatment (Figure 3). The addition of $MgCl_2$ during pre-treatment of cells with EDTA inhibited PI uptake-enhancing activity, resulting in fluorescence levels similar to those seen in cells exposed to TSP alone (Figure 4). Pre-treatment of *S. enteritidis* with $MgCl_2$ alone (in Tris-HCl) did not have any effect on the PI uptake when exposed to TSP (Figure 4). The addition of $MgCl_2$ along with TSP in the treatment solution resulted in a reduction of PI uptake compared to cells exposed to TSP alone. This reduction in

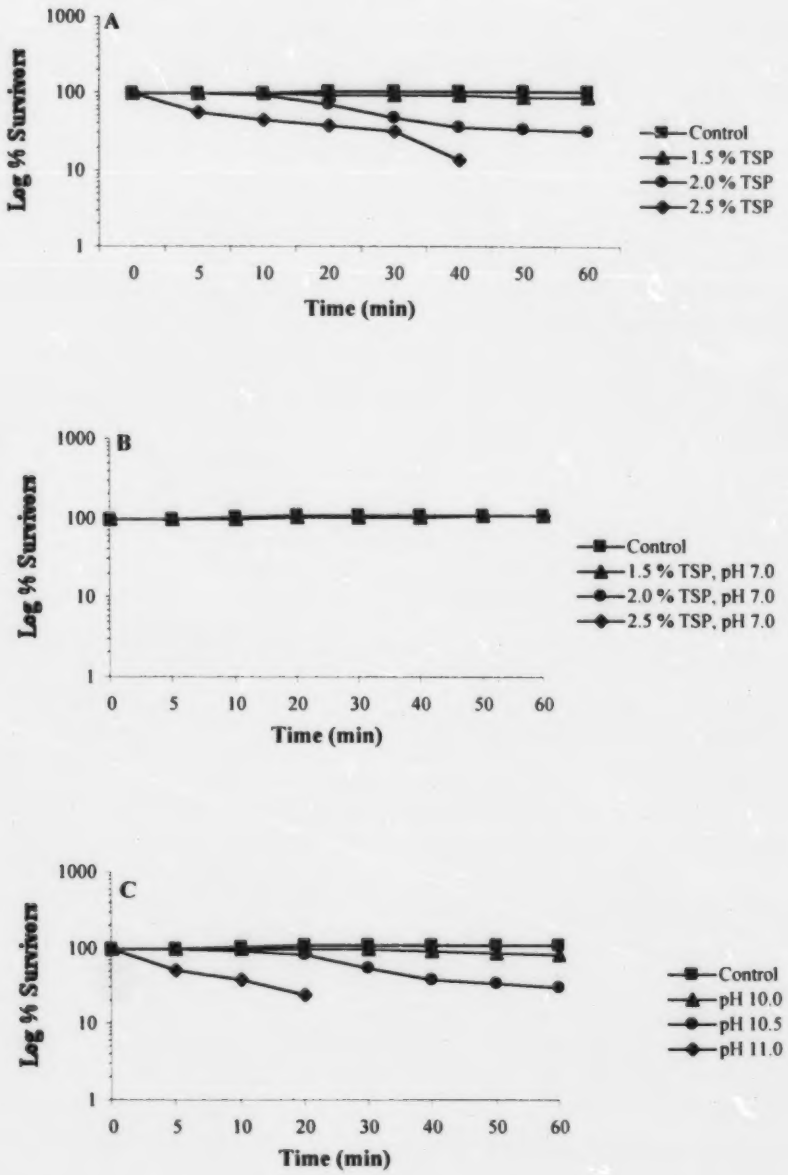



**Figure 1.** Effect of different concentrations of TSP (A), TSP pH adjusted 7.0 (B), or alkaline pH (C) on the viability of *S.* Enteritidis ATCC 4931. Colonies on TSA were enumerated after incubation at 37°C for 24 h. Data from each treatment represents means of three separate replications. The data in the graphs are presented as the percentage of surviving bacteria over time of exposure to various treatments, with the CFUs at 0 min. set to 100 %.

PI uptake was observed at 10 mM $MgCl_2$ concentration, and at concentrations of 25 mM $MgCl_2$ PI uptake was completely inhibited (Figure 5).

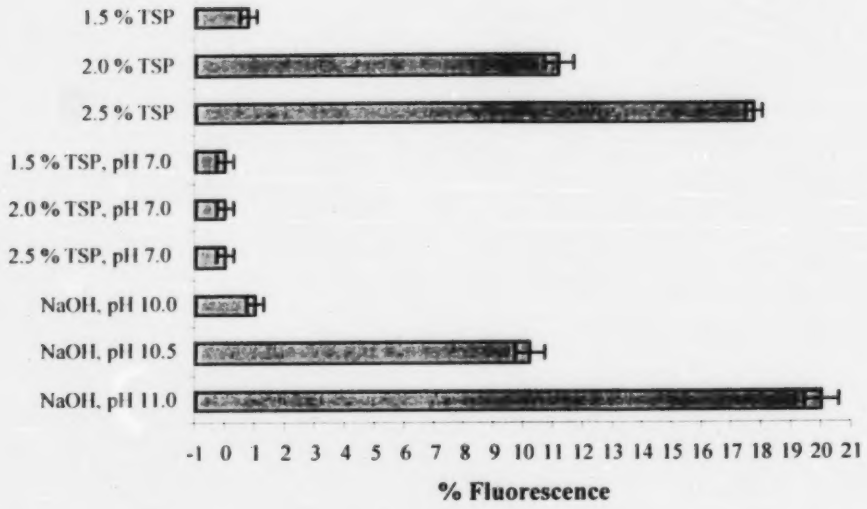



**Figure 2.** Propidium Iodide uptake by *S.* Enteritidis ATCC 4931 following treatment with different concentrations of TSP, TSP pH adjusted to 7.0, or alkaline pH, for 1 h at RT. PI uptake was measured as an increase in fluorescence relative to that of untreated cells. Percentage fluorescence values for each treatment represent means of three replications. Fluorescence values obtained for untreated cells were subtracted from all experimental values.

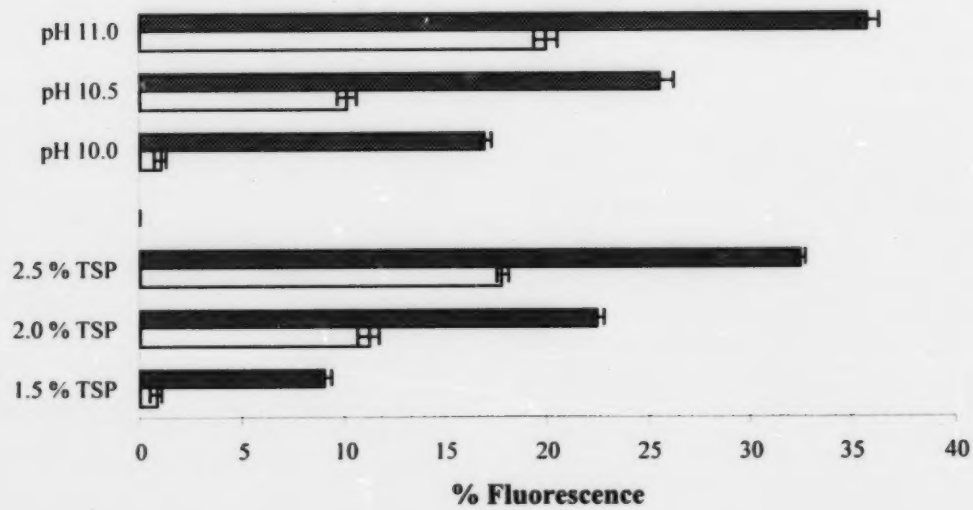



**Figure 3.** Propidium Iodide uptake by *S.* Enteritidis ATCC 4931 exposed to TSP or alkaline pH (empty bars) or pretreated with 1.0 mM EDTA in 0.1 M Tris-HCl for 30 min (filled bars) prior to 1 h treatment with TSP or alkaline pH at RT. Percentage fluorescence values for each treatment represent means of three replications. Fluorescence values obtained for untreated cells were subtracted from all experimental values.

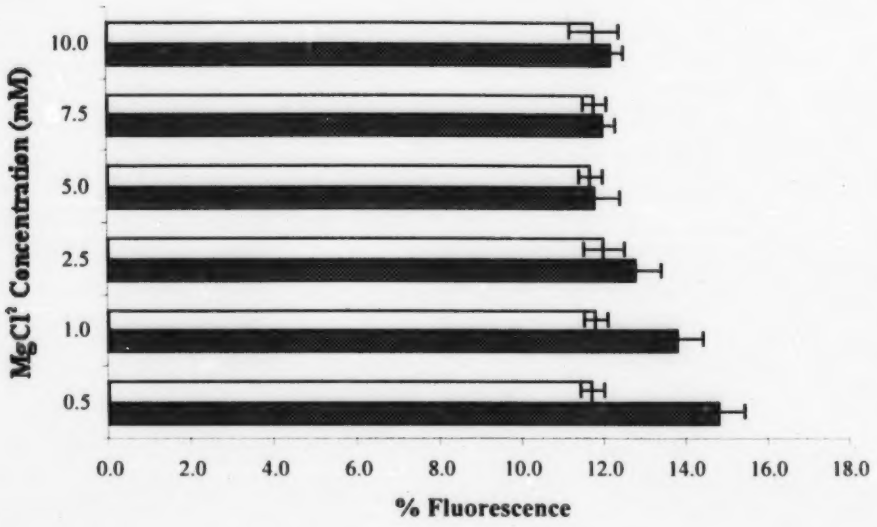



**Figure 4.** Propidium Iodide uptake by *S.* Enteritidis ATCC 4931 pretreated with different concentrations of $MgCl_2$ (empty bars) alone or in combination with 1.0 mM EDTA (filled bars) for 30 min prior to 1 h treatment with 2.0 % TSP at RT. Fluorescence values obtained for untreated cells were subtracted from all experimental values. Percentage fluorescence values for each treatment represent means of three replications.

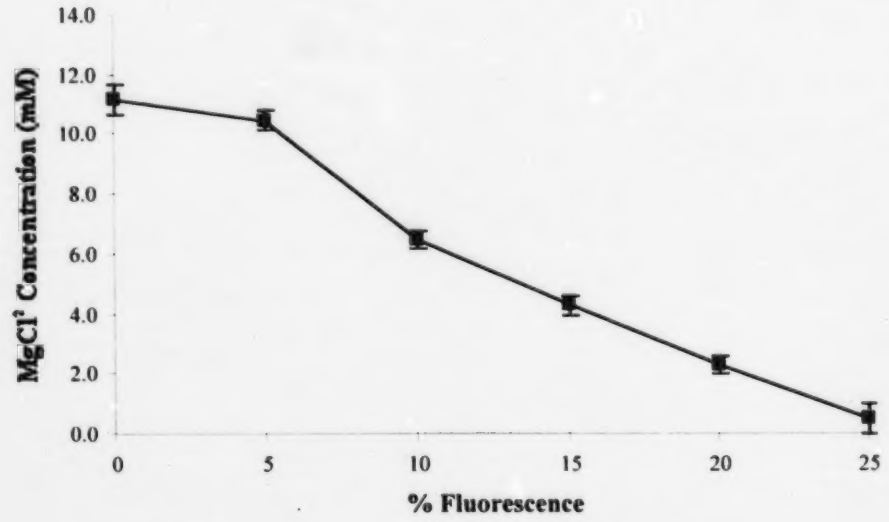



**Figure 5.** Propidium Iodide uptake by *S.* Enteritidis ATCC 4931 exposed to 2.0 % TSP along with different concentrations of $MgCl_2$ for 1 h at RT. Fluorescence values obtained for untreated cells were subtracted from all experimental values. Percentage fluorescence values for each treatment represent means of three replications.

*Osmotic response*

Cells exposed to treatment solutions containing no TSP (control) or pH 7.0 ± 0.2 solutions completely retained their ability to plasmolyse when transferred to a 0.75 M NaCl solution. When cells were exposed to 1.5 % TSP or pH 10.0, they sustained membrane damage, which was evidenced by the partial loss of ability to plasmolyse (Figure6). This loss of ability to plasmolyse increased with increasing TSP concentration or increasing alkaline pH. At concentrations of 2.5 % TSP, or pH 11.0, cells completely lost their ability to plasmolyse, resulting in a negative osmotic response (Figure 6). However, when cells were exposed to TSP treatment solutions (1.5, 2.0, 2.5 %) where the pH had been adjusted to 7.0, cells retained their ability to plasmolyse (Figure 6).

*Leakage of cellular contents*

The absorbance ($A_{260}$) of cell filtrates from cells exposed to TSP was significantly higher than control values. However, no significant difference in $A_{260}$ was observed in cell filtrates from cells exposed to different concentrations of TSP (Table 2). Increasing the time of exposure to different concentrations of TSP did not have any significant effect on $A_{260}$.

To determine whether the cell filtrate contained any nucleic acids, aliquots of concentrated filtrate from each treatment were analyzed by agarose gel electrophoresis. DNA was detected in filtrates from *S. enteritidis* exposed to all of the different concentrations TSP, whereas DNA could not be detected from control filtrates (Figure 7). Visual examination of the gel showed no significant difference in band pattern or intensity among different treatments.

Bradford assay on the cell filtrates of cells exposed to TSP revealed a very high concentration of protein, while none could be detected from control filtrates (Table 2). The protein concentration of cell filtrates exposed to TSP was concentration dependent.

*Release of LPS*

After exposure of *S.* Enteritidis to different concentrations of TSP, the same concentrations of TSP that had been pH adjusted to 7.0, or alkaline pH, cell free supernatants were processed using SDS-PAGE to investigate the possible release of LPS. Very little LPS were present in the supernatant of control preparations compared to TSP or alkaline pH-treated suspensions (Figure 8), where prominent ladder patterns characteristic of smooth-type LPS can be seen. The supernatant of cells exposed to different concentrations of TSP that had been pH adjusted to 7.0 showed very little LPS release (similar to control), compared to TSP or alkaline pH-treated suspensions.

*Electron microscopy*

The outer and cytoplasmic membranes of control cells remained intact, displaying a prominent tripartite structure and separating periplasmic space. The cytoplasm in the control cells appeared as a tightly packed, non-particulate, electron dense mass distributed throughout the cell with the chromatin present as a fine fibrillar mass in the center. Granular ribosomes (33) can be seen evenly distributed throughout the chromatin (Figure 9A). In contrast, cells exposed to TSP (Figure 9B, and C), or alkaline pH (Figure 10A, and B), appear wrinkled and collapsed. The intensity of cell wrinkling and collapse increased with exposure to increasing TSP concentration or alkaline pH. The outer membrane of cells exposed to 1.5 % TSP (Figure 9B) or pH 10.0 (Figure 10) appeared either loosely associated, disrupted or dissolved. Cross-sections of cells exposed to 1.5 % TSP (Figure 9B) or pH 10.0 (Figure 10) revealed a reduced electron dense cytoplasm that appeared granular and loosely packed. This suggests that some lysis and leakage of the cytoplasm had occurred. The chromatin in these cells was less fibrillar and less dense, with clear vacuolar regions (arrows in Figure 9B and 10A). Cells exposed to 2.5 % TSP (Figure 9C) or pH 11.0 (Figure 10B) appeared collapsed with their outer and cytoplasmic

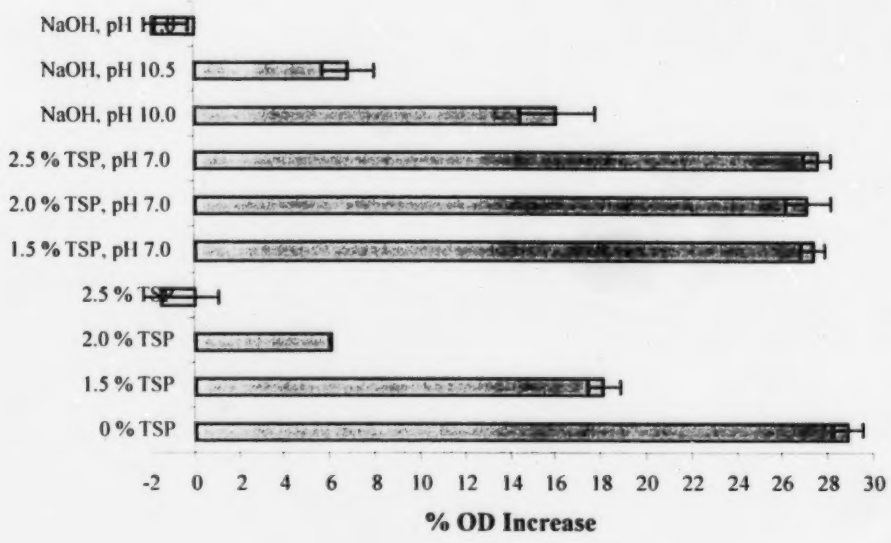

NaOH, pH H
NaOH, pH 10.5
NaOH, pH 10.0
2.5 % TSP, pH 7.0
2.0 % TSP, pH 7.0
1.5 % TSP, pH 7.0
2.5 % TSP
2.0 % TSP
1.5 % TSP
0 % TSP
-2 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
% OD Increase

**Figure 6.** Osmotic responses of *S.* Enteritidis ATCC 4931 following treatment with different concentrations of TSP, TSP pH adjusted to 7.0, or alkaline pH, for 1 h at RT. Osmotic responses of treated cells measured as the increase in OD of cell suspension placed in 0.75 M NaCl.

**Figure 7.** Ethidium bromide stained agarose gel of filtrates of *S.* Enteritidis ATCC 4931 treated with different concentrations of TSP. Lane 1- λ marker; 2- Control; 3- 0.5 % TSP; 4- 1.0 % TSP; 5- 1.5 % TSP; 6- 2.0% TSP; 7- 2.5 % TSP.

membranes disrupted, showing holes through which discharge of intracellular material occurred (Figure 11). Exposure to 2.5 % TSP or pH 11.0 showed considerable condensation of the cytoplasm into what appeared to be compacted regions of both residual ribosomes and amorphous bodies associated with the disrupted membranes (Figure 9C, and 10B). In these cells, the chromatin (which appeared granular) appeared to be diffusing out of the cytoplasm and into the surrounding environment (Figure 11C, and D). In most cases, disrupted cytoplasmic membranes could be observed in close association with the condensed cytoplasm (Figure 9C, and 10B).

### Western Blots analysis of heat shock proteins

When proteins from TSP treated and untreated *S.* Enteritidis cell populations were examined using polyacrylamide gel electrophoresis (PAGE), no differentially-induced protein bands were detected (data

not shown). Furthermore, only background (housekeeping) levels of the known heat shock proteins (HSPs), which among other things function in the protection of proteins under hostile conditions, were observed in PAGE gels (based on the molecular weights of expressed proteins). This finding was confirmed using the highly sensitive Western blot assay with antibodies against the HSPs DnaK (70Kd) and GroEL (60Kd) (Figure 12). Only background expression of GroEL or DnaK were detected in *S.* Enteritidis cells pretreated with 1.5% TSP, an alkaline solution of pH 9.8, or in untreated control populations. In contrast, heat-shocked *S.* Enteritidis cells (positive control) showed strong induction

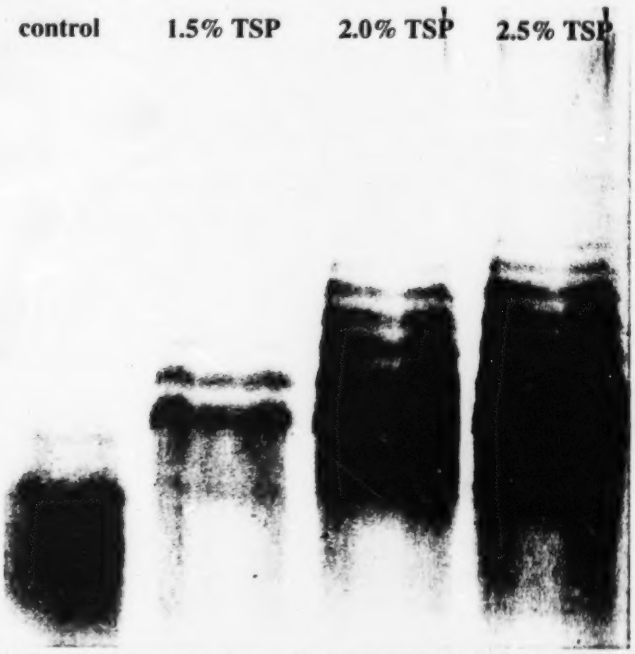



**Figure 8.** Silver-stained SDS-polyacrylamide gel (14.0 %) of proteinase K-treated filtrates of *S.* Enteritidis ATCC 4931 treated with different concentrations of TSP, TSP pH adjusted to 7.0, or alkaline pH. Lanes: 1- untreated filtrate; 2- 1.5 % TSP filtrate; 3- 2.0 % TSP filtrate; 4- 2.5 % TSP filtrate; 5- 1.5 % TSP pH adjusted to 7.0 filtrate; 6- 2.0 % TSP pH adjusted to 7.0 filtrate; 7- 2.5 % TSP pH to 7.0 filtrate; 8- pH 10.0 with NaOH filtrate; 9- pH 10.5 with NaOH filtrate; 10- pH 11.0 with NaOH filtrate.

of the GroEL HSP (but no difference in levels of production of DnaK), providing evidence that the protection afforded to TSP pretreated cells was not based on the synthesis of any known HSPs (Figure 12).

***2D gel electrophoresis protein analysis***

Two-dimensional gel electrophoresis was used to resolve the total cellular proteins of *S.* Enteritidis treated with 1.5 % TSP for 1 h at room temperature. Approximately 300 and 250 protein spots were resolved in control (Figure 13A) and TSP (Figure 13B) treated cells, respectively. Approximately 8 proteins were induced and 11 proteins were repressed more than 2-fold by TSP treatment. Of the approximately 300 spots expressed in control cells, 8 spots were unique to control cells compared to only 1 spot as a result of TSP treatment. Studies are currently underway to determine the molecular weight and isoelectric points of the various unique, induced and repressed proteins in both the control and TSP treated cells. Studies are also being undertaken to identify these proteins by MS/MS.

The 2D PAGE of whole-cell protein extracts of 12 h planktonic and biofilm *S.* Enteritidis cells permitted the separation of approximately 270 and 252 distinct protein spots, respectively. Although the overall pattern of protein spots were similar (i.e., the number and position of the spots) between gels from each biological state, distinct differences could be observed. 5 proteins (boxed, Figure 14B) were synthesized as a result of surface attachment. Eighteen proteins (boxed in Figure 14A) unique to the planktonic profile represented proteins that appeared to be absent in biofilm cells. As many as 17 proteins showed at least a 2-fold increase in expression in biofilm cells while as many as 54 proteins were repressed compared to planktonic cells.

Outer membrane proteins were also analyzed using 1-D PAGE gel analysis. It was found that when cells were treated with pH 7.0 buffered 1.5% TSP, the protein patterns were identical to that of the control cells (Figure 15). When pH 10 NaOH was used, the protein pattern was the same as for 1.5% TSP, although a number of unique outer membrane proteins were expressed. This indicates that at the level of the outer membrane, the effect was primarily pH driven, but that detectable changes were occurring.

| TSP Treatment (% w/v) | $A_{260}$ nm[a] | Protein Concentration (μg/μl)[b] |
|---|---|---|
| 0.0 | 0.12 | 0.00 |
| 0.5 | 1.96 | 1.57 |
| 1.0 | 1.98 | 1.99 |
| 1.5 | 1.98 | 2.82 |
| 2.0 | 1.98 | 3.11 |
| 2.5 | 1.98 | 3.12 |

**Table 2.** Effect of different concentrations of TSP on the absorbance at 260 nm and protein content of cell free filtrates of *S.* Enteritidis ATCC 4931. Data are means of three separate replications. [a] Absorbance values from the control treatment (0 % TSP) was subtracted from the treatment values. [b] Protein content estimated by Bradford assay.

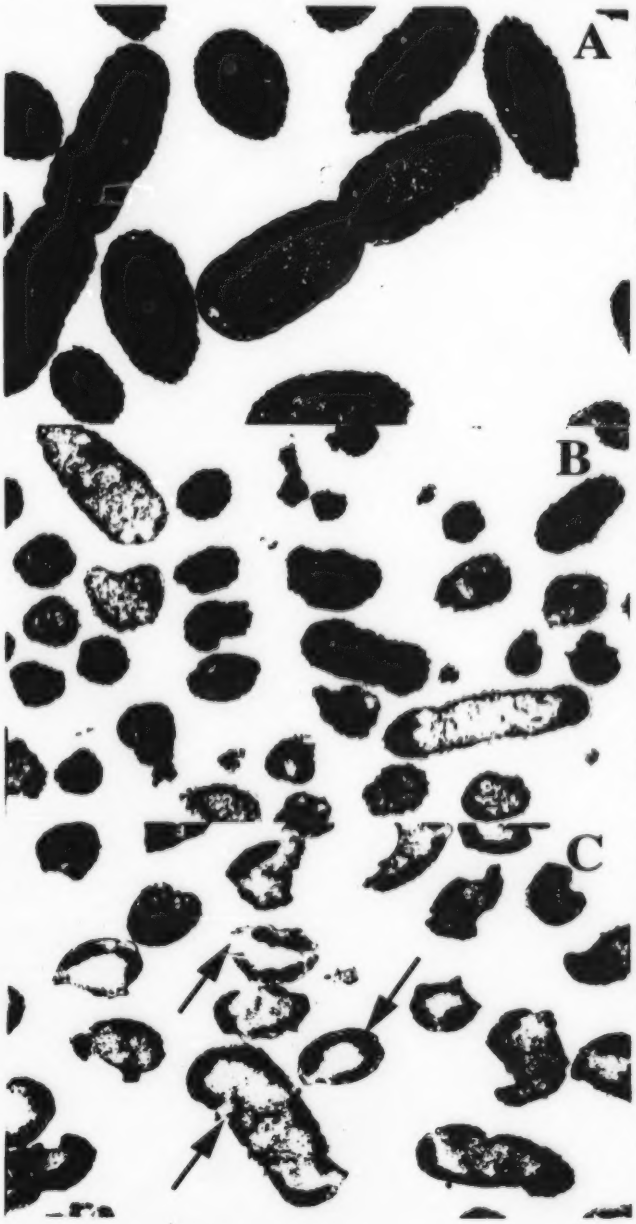



**Figure 9.** Electron micrographic thin sections of untreated control *S.* Enteritidis ATCC 4931 cells (A), cells exposed to 1.5 % TSP (B), and cells exposed to 2.5 % TSP (C). Arrows in panel B identify clear vacuolar regions. In panel C, arrows identify: a) disruption and holes in the outer and cytoplasmic membranes (the two lower left hand arrows), and b) condensation of the cytoplasm into what appeared to be compacted regions of residual ribosomes and amorphous bodies situated close to the disrupted membrane (the upper right hand arrow).

### *Discussion*

The results presented in this study support the conclusion that TSP is a potent OM-disintegrating agent, as evidenced by its ability to release LPS, nucleic acid, intracellular proteins as well as direct EM evidence. Indirect measures of TSP effects on the OM of *S. enteritidis* include an increase in PI uptake and decrease in osmotic responsiveness, further suggesting that TSP causes the permeabilization and disruption of the cytoplasmic membrane leading to loss of cell viability. When TSP is dissolved in water or culture medium, a very alkaline pH results, however the pH of TSP in culture medium is approximately 2 units lower than that of the corresponding pH in distilled water. Trisodium phosphate at concentrations of 1.5, 2.0 and 2.5 % in TSB resulted in pH values of 10.0, 10.5 and 11.0 respectively.

In this study, *S.* Enteritidis ATCC 4931 showed death rates that varied with the concentration of TSP (Figure 1A). These death rates were comparable to those obtained when cells were exposed to solutions adjusted using

**Figure 10.** Electron micrographic thin sections of *S.* Enteritidis ATCC 4931 cells exposed to TSB pH adjusted to 10.0 (A) and 11.0 (B) using NaOH. Arrows in panel A show clear vacuolar regions. In panel B, the upper left hand arrow shows the compaction of ribosomes and amorphous bodies near the disrupted membrane, whereas the lower right hand arrow shows an example of the disrupted cytoplasmic membrane closely associated with the condensed cytoplasm.

NaOH to corresponding pH (and not TSP). *S.* Enteritidis ATCC 4931 exhibited good survival at a pH of 10.0 for an hour (Figure 1B). However, increasing the concentration of TSP in the treatment solution resulted in a rapid loss of cell survival. A similar loss in survival was observed when cell suspensions were exposed to high pH made with NaOH (Figure 1 A&B). Table 1 shows the D-values for acclimated or unacclimated (control) *S.* Enteritidis cells exposed to either 2.5% TSP, pH 11, 55°C, pH 2.6, or 30 mM $H_2O_2$. It can be seen from the D-value data that significant ($P<0.01$) cross-protection from sublethal TSP treatment was provided to cells subsequently treated with heat, high pH solutions and 2.5% TSP. Adjusting the pH of the treatment solution containing different concentrations of TSP to 7.0 results in a total elimination of antimicrobial activity of TSP (Figure 1C). Thus an indirect correlation existed between cell viability and TSP concentrations or alkaline pH.

Gram-negative bacteria are more susceptible to high pH because of their very thin peptidoglycan layer, which is only about 2 to 3 nm thick (Murray *et al.*, 1965). The peptidoglycan layer stabilizes the cytoplasmic membrane of intact bacterial cells against turgor pressure exerted by the cytoplasm (Csonka *et al.*, 1991). Therefore, the very thin peptidoglycan layer of gram-negative bacteria may be less capable of preventing the cytoplasmic membrane from bursting once it is weakened by high pH. Mendonca *et al.* (1994) have showed this weakening and bursting of the cytoplasmic membrane is caused by high pH. During this present study, *S.* Enteritidis exposed to increasing concentrations of TSP showed an increase of uptake of the membrane-impermeant fluorescent dye PI suggesting that TSP treatment led to the permeabilization of the cytoplasmic membrane. Pre-treatment of *S.* Enteritidis with EDTA followed by exposure to TSP resulted in an increased PI uptake relative to cells not pre-treated with EDTA. EDTA has a profound effect on the OM permeability barrier of gram-negative bacteria (Leive, 1974). It removes, by chelation, stabilizing divalent cations like $Mg^{2+}$ and to a lesser extent $Ca^{2+}$ from their binding sites in LPS thereby making the OM more permeable to agents that are otherwise less permeable (Helander and Vaara, 1987; Leive, 1974; Lugtenberg and van Alpen, 1983; Nikaido and Vaara.,1985.). Short treatments with EDTA have been used to introduce macromolecules and hydrophobic compounds through the OM without affecting cell viability (Helander *et al.*, 1996; Leive, 1974; Scudamore *et al.*, 1979; Stevens *et al.*, 1992). Exposing *S.* Enteritidis to EDTA alone did not lead to increased PI uptake (data not shown). However, addition of excess $MgCl_2$ during EDTA pre-treatment resulted in the inhibition of PI uptake-enhancing activity of EDTA. This observation could be attributed solely to chelation of excess $Mg^{2+}$ with EDTA, thus making EDTA unavailable for the chelation of $Mg^{2+}$ in the OM and causing increased OM permeability. Similarly including excess $MgCl_2$ during TSP exposure completely abolished the PI uptake caused by TSP (Figure 5). This could be either due to excess $MgCl_2$ directly complexing with TSP, thus making it unavailable to act on the OM of *S.* Enteritidis , or $MgCl_2$ stabilizing the OM of *S.* Enteritidis, making it resistant to TSP. How-

ever, the former explanation seems more likely as pre-treatment with $MgCl_2$, unlike EDTA, had no effect on the *uptake* of PI during TSP exposure.

Cells exposed to 2.5 % TSP, or a NaOH solution with a pH of 11.0, showed a very rapid loss of viability and exhibited the highest PI uptake. In comparison, cells exposed to 1.5 % TSP or a NaOH solution with a pH of 10.0, showed very little loss of viability and the least uptake of PI. These results suggest that there exists a clear relationship between membrane permeabilization and viability.

The increase in OD observed when cell suspensions were placed in hypotonic salt solutions,

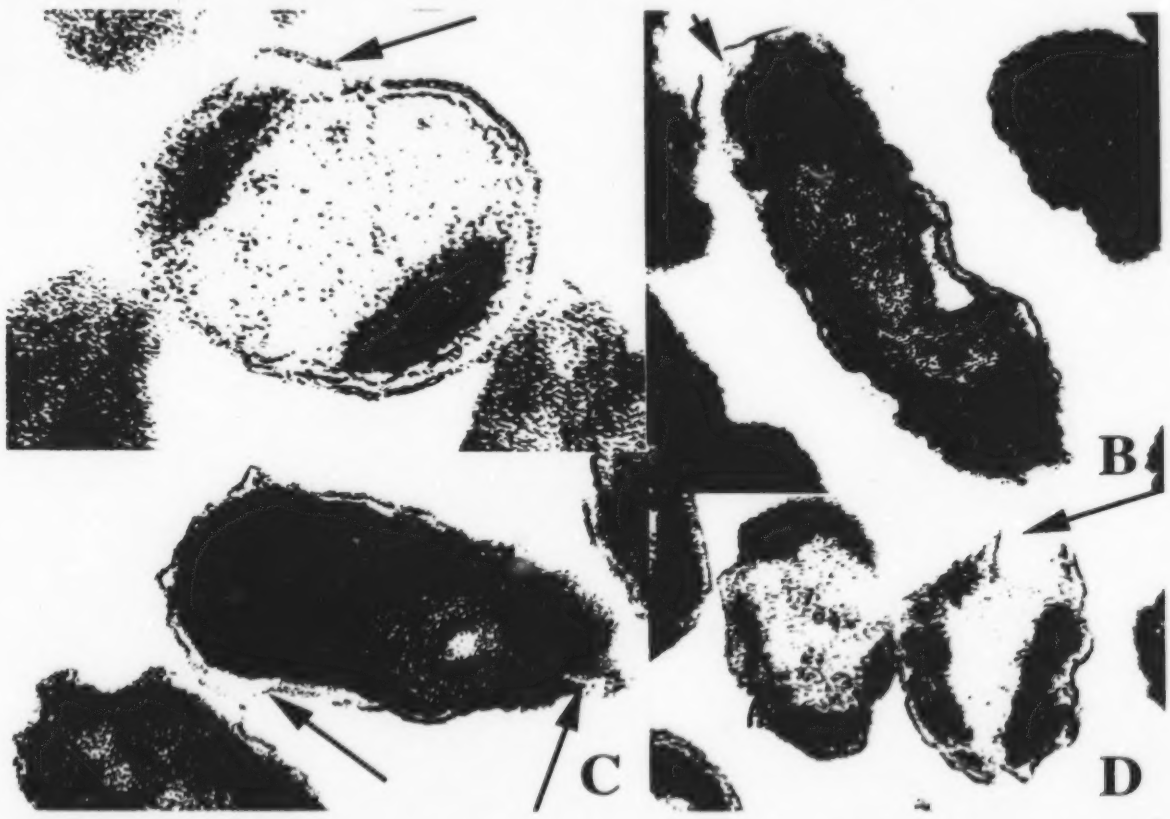



**Figure 11.** Electron micrographic thin sections of *S.* Enteritidis ATCC 4931 cells exposed to either TSB pH adjusted to 11.0 (A, B) using NaOH, or 2.5 % TSP (C, D). In all panels, arrows point to the disrupted outer and cytoplasmic membranes and concurrent discharge of intracellular contents.

known as an optical effect, is caused by an increase in light scattering that is related to the extent of plasmolysis (Mager *et al.*, 1956). In cold-shocked cells of *Klebsiella aerogenes*, there was a good correlation between loss of osmotic response and cell death (45). Korber et al. (Korber et al., 1996) developed a microscopic method for measuring palsmolysis in single cells, and demonstrated that loss of osmotic response was associated with cell death. During the present study, *S.* Enteritidis cells exposed to increasing concentrations of TSP lost their ability to plasmolyse in 0.75 M NaCl in a TSP concentration dependent manner, suggesting that the cells have sustained membrane damaged. A similar response was observed when cells were exposed to high pH made with NaOH. Thus there existed an indirect correlation between cell viability and membrane damage, as revealed by PI uptake and osmotic response.

Membrane damage due to TSP treatment was consistent with the release of LPS (Figure 8), and higher absorption at 260 nm of cell free filtrates. The absorption spectra for nucleic acids have a

maximum at 260 nm (Ulitzur, 1972); therefore 260 nm AM released by the TSP or high pH treated cells likely consisted of nucleic acids. Further support for membrane damage by TSP or high pH was provided by the demonstration of DNA and proteins in the sterile filtrates from treated cells.

Destruction of *S.* Enteritidis by disruption of the membranes was also supported by electron microscopy results. Trisodium phosphate or high pH appeared to have three obvious effects: (i) wrinkling and collapse of cells (Figure 9B, C, and 10); (ii) disruption of the membranes and leakage of

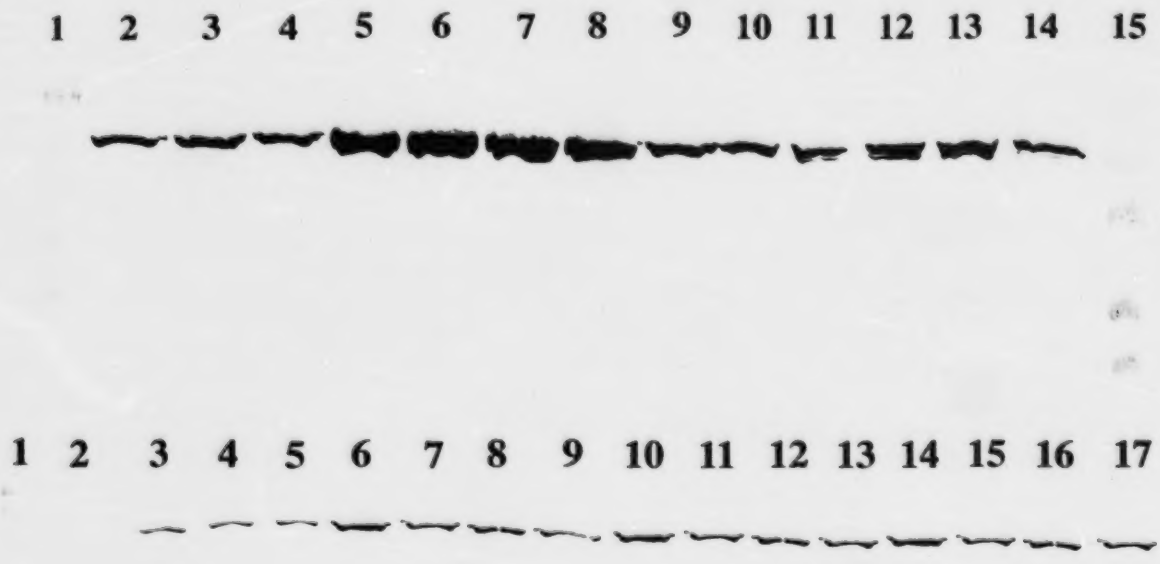



**Figure 12.**

**TOP.** Western immunoblot of the one dimensional SDS-PAGE profiles of intracellular proteins of *S.* Enteritidis subjected to different stresses using Anti-GroEL monoclonal antibody. **Lane 1**- Kaleidoscope molecular weight marker; **2**- Control log phase cells; **3**- Control late log phase cells; **4**- Control early stationary phase cells; **6**- Heat shocked (42°C) log phase cells; **7**- Heat shocked (42°C) late log phase cells; **7**- Heat shocked (42°C) early stationary phase cells; **8**- Heat shocked (42°C) stationary phase cells; **9**- pH 9.8 log phase cells; **10**- pH 9.8 late log phase cells; **11**- pH 9.8 early stationary phase cells; **12**- 1.5% TSP log phase cells; **13**- 1.5% TSP late log phase cells; **14**- 1.5% TSP early stationary phase cells; **15**- Broad range molecular weight marker.

**BOTTOM.** Western immunoblot of the one dimensional SDS-PAGE profiles of intracellular proteins of *S.* Enteritidis subjected to different stresses using Anti-DnaK monoclonal antibody. **Lane 1**- Kaleidoscope molecular weight marker; **2**- Control log phase cells; **3** – Control late log phase cells; **4**- Control early stationary phase cells; **5**- Control stationary phase cells; **6**- Heat shocked (42°C) log phase cells; **7**- Heat shocked (42°C) late log phase cells; **8**- Heat shocked (42°C) early stationary phase cells; **9**- Heat shocked (42°C) stationary phase cells; **10**- pH 9.8 log phase cells; **11**- pH 9.8 late log phase cells; **12**- pH 9.8 early stationary phase cells; **13**- pH 9.8 stationary phase cells; **14**- 1.5% TSP log phase cells; **15**- 1.5% TSP late log phase cells; **16**- 1.5% TSP early stationary phase cells; and **17**- 1.5% TSP stationary phase cells.

internal contents (Figure 9C, and 11); and (iii) condensation and localization of the cytoplasm close to the membrane (Figure 9C, 10B, and 11D). This condensation and localization of the cytoplasm close to the membrane may be explained by the hydrophilic nature of DNA. DNA is highly negatively charged and water soluble at high pH, which aids its extraction from cells (Sambrook *et al.*, 1989). Other cytoplasmic molecules such as proteins would denature and precipitate at high pH. Under this condition, DNA would separate as a liquid phase from the rest of the cytoplasm and would be released out of the cell once the outer and cytoplasmic membranes are disrupted (arrows in Figure 9C, 11C, and

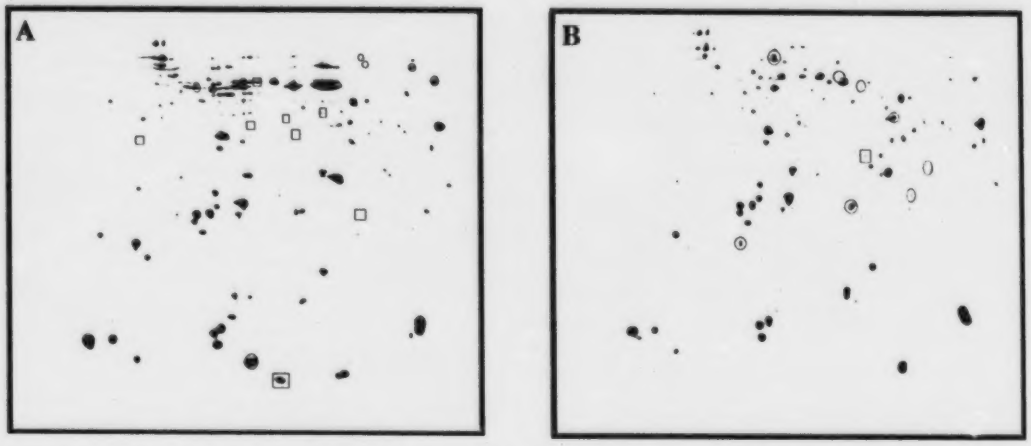



**Figure 13A,B.** Silver stained 2-D PAGE gels of untreated *S.* Enteritidis (A) and *S.* Enteritidis cells (B) treated with 1.5% TSP for 1 hr at room temperature. Spots marked with a circle indicate proteins which have undergone 2-fold or greater induction as compared with the other treatment. Spots marked with a square are unique proteins seen only in either of the two treatments (control or TSP treated). Each image is the aligned composite of not less than 5 replicate 2-D PAGE gels. Alignment and differential imagery was performed using PD-Quest software (BioRad).

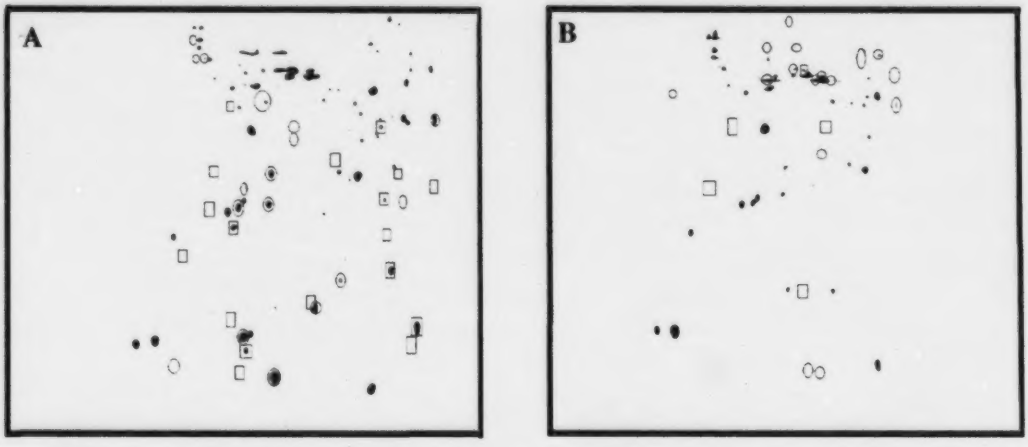



**Figure 14A,B.** Silver stained 2-D PAGE gels of planktonic *S.* Enteritidis (A) and *S.* Enteritidis biofilm cells (B). Spots marked with a circle indicate proteins which have undergone 2-fold or greater induction as compared with the other treatment. Spots marked with a square are unique proteins seen only in either of the two treatments (control or TSP treated). Each image is the aligned composite of not less than 5 replicate 2-D PAGE gels. Alignment and differential imagery was performed using PD-Quest software (BioRad).

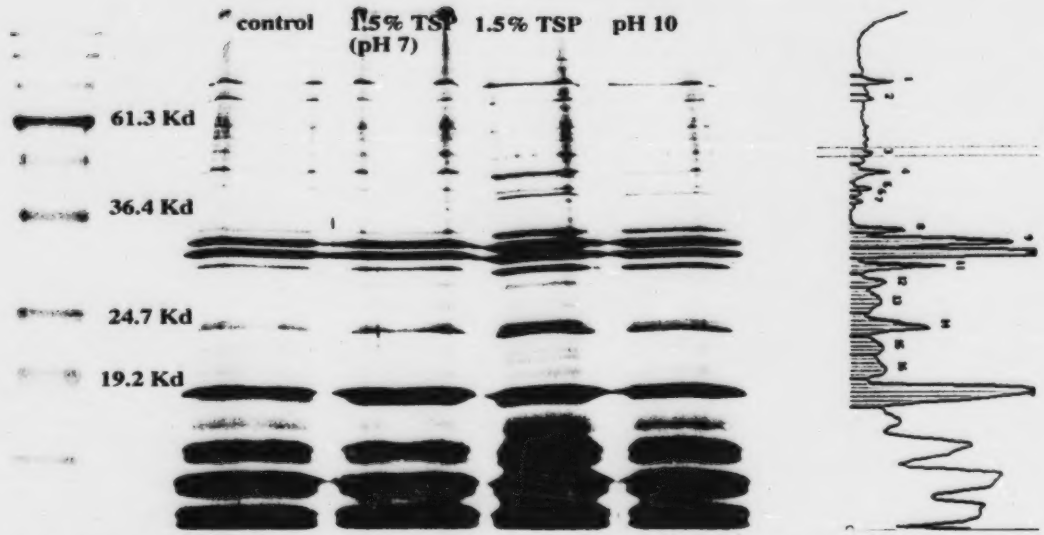



**Figure 15.** Silver stained 1-D PAGE gel of *S.* Enteritidis outer membrane proteins. A number of differentially expressed outer membrane proteins were quantified when the cells were treated with either 1.5% TSP or pH 10 NaOH. In both cases, the band patterns were equivalent. Note the example chart tracing on the right hand of the gel providing an example of the relative intensities of each band contained in lane 2 (1.5% TSP at pH 7). Numeric data (intensity and area under the curve) was obtained for all bands identified using image analysis, facilitating comparisons between treatments.

D). This would explain the increased absorbance at 260 nm of filtrates of cells exposed to TSP or high pH (Table 2). Similar reports of membrane damage, leakage of internal content and condensation of the cytoplasm have been reported by electron microscopic studies in *Salmonella* exposed to high hydrostatic pressure (Mackey *et al.*, 1994) and in *Listeria monocytogenes* exposed to freezing and thawing (Dykes, 1999; El-Kest and Marth, 1992). Overall, these results support the view that high pH during TSP treatment along with sequestration of metal ions in the outer membrane by TSP, led to membrane damage, release of intracellular components and cell death.

Despite the fact that the development of acquired resistance to alkaline stress didn't involve the induction of known proteins (i.e., the HSPs GroEL or DnaK) (Figure 12), there is still significant evidence that a protein based resistance mechanism is operative. A large number of cytoplasmic proteins underwent a >2 fold change in the levels of expression of certain proteins (Figure 13). Figure 15 indicated that differential expression of outer membrane proteins may also play an important role in the response and survival of these bacteria. While it is known that biofilms of *S.* Enteritidis differ from planktonic *S.* Enteritidis cells in terms of expression of unique proteins (Figure 14), the impact this has on acquired resistance to stress factors like TSP and other alkaline agents remains unclear. This remains a focal point in research ongoing at the University of Saskatchewan.

The identify of these proteins at present remain unknown, however this element of this study remains ongoing. Individual proteins, as represented by individual spots on the 2-D PAGE gels shown in Figures 13 and 14 will be identified using MS/MS analysis, and their amino acid sequences will then be compared with proteomic databases to determine whether homologous proteins in other microbial species might play a similar role. Furthermore, the genetic regulatory elements may be better understood in other species, thereby facilitating the design of engineered antimicrobial strategies of value to the food industry, and the meat processing sector in particular.

*Carbon source utilization of* **S. enteritidis** *cells treated with sub-lethal doses of TSP.*

We were also interested in how the metabolic potential of TSP-treated cells changed; these changes may ultimately reveal the effect of the proteome level changes seen in the 2-D PAGE analyses. Accordingly, phenotypic arrays were compared using BIOLOG GN plates. *S.* Enteritidis cells sensitized to TSP were obtained using the methods described previously for protein analyses. For determinations of carbon source utilization, cells were resuspended in PBS after 2x rinsing of TSB and TSP from the cells. Cells were then diluted 1/10 to minimize carry-over of carbon (and also reduce the concentration of cells) which could potentially interfere (result in false positives). A 150 $\mu$l aliquot of control or treated cells was then added to each of the 96 wells of the Biolog plate. Similarly, washed *S.* Enteritidis cells were resuspended in PBS amended with 0.5% TSP, and dispensed directly to the Biolog plates. Biolog GN plates contain 95 different sole carbon sources and a no-carbon control arrayed in the 96-well format. Each well also contains tetrazolium salt as a colorimetric indicator. The microtiter plates consisted of several groups of carbon sources, including simple carbohydrates, polymers, esters, carboxylic acids, brominated compounds, amides, amino acids, aromatic compounds, amines, alcohols, and phosphorylated compounds.

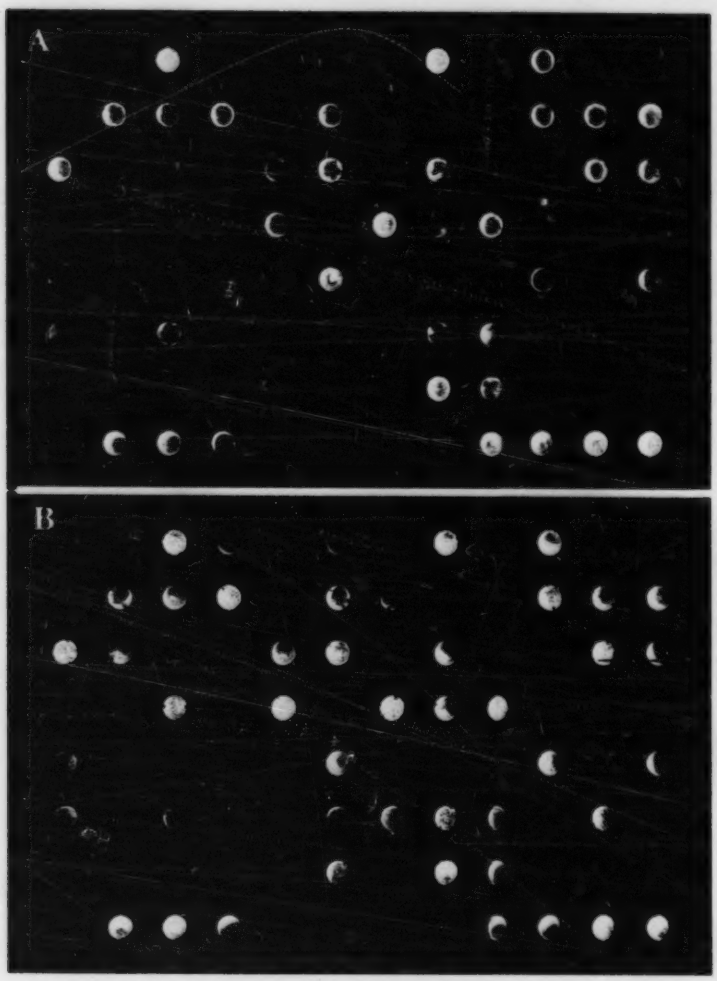



**Figure 16.** Difference images of sole carbon source utilization profiles of *S.* Enteritidis cells. When cells were pretreated with 0.5% TSP, they lost the ability to utilize glycyl L- aspartic, glycyl L-glutamic, acetic and cis-aconitic acids (to varying degrees) when compared with the untreated control(seen as the red wells). In contrast, when *S* Enteritidis cells were pre-treated with 1.5% TSP for 1 hr, a number of differences from that of the control phenotypic array were observed (Figure 16B).

Microtiter plates were incubated 22°C for either 6 or 24 hours before using image analysis to quantify color development in the wells. The image analysis procedure involved acquiring images using a Hewlett Packard flatbed scanner 6100C/T (equipped with transparency adapter), after which analysis of the intensity of color development using Scion Image™ (NIH) software. Pixel grey level values (ranging from 0 = white to 255 = black) for positive wells were defined by using the value of the control well as threshold. Grey level images of control and treatment plates were also pseudocolored (control=red; treatment=green) and combined digitally, providing a direct visual method for identifying wells where carbon utilization patterns have become altered or changed from control reactions. Wells in combined images thus appeared bright yellow green (indicating an equivalent reaction in both control and treatment wells), red (indicating wells where the treatment has resulted in the loss in the ability to utilize these substrates), or green (indi-

cating newly acquired metabolic activity). Note that light colors indicate poor utilization of a particular carbon source. It was observed that *S.* Enteritidis cells suspended in 0.5% TSP lost the ability to utilize glycyl L- aspartic, glycyl L-glutamic, acetic and cis-aconitic acids (to varying degrees) when compared with the untreated control. Figure 16A shows the difference image highlighting these wells (D1 and D2; F11 and F12) differences between these treatments. In contrast, *S. enteritidis* cells pretreated with 1.5% TSP for 1 hr showed a number of differences from that of the control phenotypic array (Figure 16B), and included only D1, D2 and weak F12 from the previous comparison using 0.5% TSP. The effective utilization of a number of carbon sources appeared to be reduced from that of the control cells, suggesting a possible medium-term (24 hr) change in membrane permeability or metabolic capability resulted from TSP stress. The length of time which cells remain resistant to subsequent stress factors for sub-lethal stress sensitization is clearly of importance.

Overall, the differences between reactions for the control and treatment cell populations are somewhat difficult to compare, however it is hoped they reflect an important change in cell metabolic function. It remains to be shown at the molecular/protein level what these changes represent. MS analysis of proteins will hopefully lead to connections between these data.

*Study area 3. Efficacy enhancement of other food-grade antimicrobial agents.*

**Project objective.**

While a number of different antimicrobial treatments were tested against bacteria on meat disk over the course of this study (e.g., TSP at various concentrations, PDP-TSP, PDP-2x-TSP, and nisin used as a permeabilizing agent for TSP), the results were generally not equivalent to those observed in the flow cell model system. The primary reason for this is thought to be the pH dependent mode of action of TSP. Thus, the buffering microenvironment of the meat disk model system resulted in a rapid return of the system pH to sub-inhibitory concentrations. It is also possible that plasmolysis on meat disks did not occur as efficaciously as in the flow cell model system. Unfortunately, since we could not directly monitor plasmolysis on the surfaces of meat disks, the potential impact of this factor remains as yet undetermined. In the case of nisin, other unknown factors appear to play an inhibitory role on meats despite indications in pure culture flow cell studies that efficacy enhancement using nisin as a permabilizing agent for later TSP treatment. A number of treatments were employed in the flow cell model system which were not attempted on meat disks, including, for example, treatment of biofilm bacteria with PDP-myristyl-L-methionine or organic acids. Since promise was not shown with some of these approaches, they were not persued.

**Summary of results.**

Some investigations performed included using the PDP process in conjunction with nisin, a membrane-permeabilizing peptide antimicrobial agent. Findings in the flow cell model system indicated that there may be a synergistic effect when PDP was applied prior to nisin. While nisin did inhibit the gram positive isolates *Brochothrix thermosphacta* and *Listeria monocytogenes* (which were quite nisin sensitive) no combinatorial effect between nisin and the PDP process was seen when examined on meat tissue. The gram positive organisms *L. monocytogenes* and *B. thermosphacta* both exhibited initial nisin sensitivity at fairly low concentrations (from 0.01 to 0.1 g $L^{-1}$ for both strains) when present on fatty or lean meat tissue. Even larger log reductions in the number of viable bacteria per $cm^2$ meat were recorded for these 2 strains when 1.0 g $L^{-1}$ nisin was applied for a 3 minute period. Under

**Table 1.** Effect of various concentrations of nisin and 1.5 M NaCl on *L. monocytogenes* on pork tissue.

**Log bacteria/$cm^2$**

| | Fat TSA + 0.6% YE | | Lean TSA + 0.6% YE | |
|---|---|---|---|---|
| **Initial** | 6.04 | | 5.72 | |
| **treatment** | **Log** | **Log Difference** | **Log** | **Log Difference** |
| none | 6.04 | - | 5.72 | - |
| Bacteria and $H_2O$ | 5.99 | -0.05 | 5.64 | -0.08 |
| Bacteria and NaCl | 5.96 | -0.08 | 5.73 | 0.01 |
| Bacteria and 0.01 g $L^{-1}$ nisin | 4.97 | -1.07 | 5.61 | 0.11 |
| Bacteria and 0.1 g $L^{-1}$ nisin | 2.10 | -3.94 | 4.75 | -0.97 |
| Bacteria and 1.0 g $L^{-1}$ nisin | 1.44 | -4.60 | 2.56 | -3.16 |
| Bacteria and 0.1 g $L^{-1}$ nisin and 1.5 M NaCl | 4.50 | -1.54 | 5.24 | -0.48 |

**Table 2.** Effect of various concentrations of Nisin and 1.5 M NaCl on *B. thermosphacta* on pork tissue.

| | Log bacteria/cm² | | | |
|---|---|---|---|---|
| | Fat | | Lean | |
| | TSA + 0.6% YE | | TSA + 0.6% YE | |
| **Initial** | 5.45 | | 4.72 | |
| **treatment** | **Log** | **Log Difference** | **Log** | **Log Difference** |
| none | 5.45 | - | 4.72 | - |
| Bacteria and $H_2O$ | 5.01 | -0.44 | 4.42 | -0.30 |
| Bacteria and 0.001 g $L^{-1}$ nisin | 5.22 | -0.23 | 4.02 | -0.70 |
| Bacteria and 0.01 g $L^{-1}$ nisin | 4.96 | -0.49 | 4.12 | -0.60 |
| Bacteria and 0.1 g $L^{-1}$ nisin | 1.45 | -4.00 | 2.64 | -2.08 |
| Bacteria and 0.01 g $L^{-1}$ nisin and 1.5 M NaCl | 5.17 | -0.28 | 5.11 | -0.39 |

these conditions, *L. monocytogenes* (Table 1) underwent a 4.6 log reduction in cell numbers when on fatty tissue, and an ~3.2 log reduction when on lean tissue. Under similar conditions, *B. thermosphacta* underwent a 4.0 log reduction (Table 2) on fatty tissue and an ~ 2.0 log reduction on lean tissue. It is noteworthy that sub-optimal inhibitory nisin concentrations were employed such that when nisin was applied in conjunction with PDP, synergistic effects would easily be detected. We subsequently focused on the enhancement of nisin activity at 0.1 mg $L^{-1}$ for both these strains. Theinitial experiments conducted using PDP enhancement (Tables 1 and 2) inadvertently saw the simultaneous addition of 1.5 M NaCl PDP solution and nisin. Thus, cells were not exposed to these compounds during cell deplasmolysis, resulting in a protective effect (Tables 1+2, last row) following 0.1 g $L^{-1}$ nisin treatment. Repeated experiments with nisin applied in sequence failed to improve the efficacy of nisin

**Table 3.** Effect of 0.1 g $L^{-1}$ Nisin and 1.5 M NaCl on *Pseudomonas* D23 on pork tissue.

| | Log bacteria/cm² | | | |
|---|---|---|---|---|
| | Fat | | Lean | |
| | TSA + 0.6% YE | | TSA + 0.6% YE | |
| **Initial** | 4.65 | | 4.36 | |
| **treatment** | **Log** | **Log Difference** | **Log** | **Log Difference** |
| none | 4.65 | - | 4.36 | - |
| Bacteria and $H_2O$ | 4.98 | 0.33 | 4.41 | 0.05 |
| Bacteria and NaCl | 3.87 | -0.78 | 3.89 | -0.47 |
| Bacteria and 0.1 g $L^{-1}$ nisin | 4.96 | 0.31 | 4.08 | -0.28 |
| Bacteria and 1.0 g $L^{-1}$ nisin | 4.90 | 0.25 | 3.99 | -0.37 |
| Bacteria and 2.5 g $L^{-1}$ nisin | 4.82 | 0.17 | 3.97 | -0.39 |
| Bacteria + 2.5 g $L^{-1}$ nisin and NaCl | 4.31 | -0.34 | 3.53 | -0.83 |

against these bacteria. Likewise, the use of nisin as a permeabilizer to be followed by TSP treatments also failed to result in efficacy enhancement on meat surfaces. It has previously been reported that the activity of nisin and other bacteriocin compounds may be sensitive to the presence of chelators or high ion concentrations. High concentrations of TSP may thus have impacted the potential for nisin to remain in a functional conformation. The knowledge of this potential limitation to this method as it might relate to industrial practice, is quite valuable.

Sample data is also provided where the meat isolate, *Pseudomonas* D23 was challenged with nisin, as well as nisin in combination with salt (Table 3). These results contrast observations in flow cells with gram negative bacteria (where increased nisin efficacy was seen; data not shown), as the viability of *Pseudomonas* D23 was unaffected by even very high concentrations of nisin (2.5 g $L^{-1}$). One possible explanation for the apparent increase in the number of "killed" cells in flow cell studies may reflect the respective modes of action of nisin and the fluorescent viability indicator. Nisin is an amphipathic (dual-natured) cationic peptide which has both hydrophobic and hydrophylic character. As a consequence, the hydrophobic ends of nisin tend to become inserted in the lipid layers of microbial membranes. In gram positive bacteria, this insertion causes cell permeabilization such that the cell becomes leaky, the transmembrane gradients diminishes, and the cell dies. In gram negative bacteria, a layer of lipopolysaccharide overlays outer and inner cell membranes. Nisin therefore cannot directly span the both cell membranes and hence typically do not cause significant reduction in viability in most gram negative bacteria. Fluorescent viability probes such as the Live/Dead probe (Molecular Probes, Inc., Eugene, Oregon) are sensitive to the condition of the cell's membranes; this particular stain includes a membrane permeable nucleic acid stain (Syto 9) which fluoresces green upon intercalation with nucleic acids, as well as a membrane impermeant nucleic acid stain (propidium iodide) which fluoresces red if able to reach the cell's nucleic acids. In the case of nisin-treated gram negative bacteria, it is hypothesized that nisin permeabilized the outer membrane and permited a higher concentration of the low molecular weight propidium iodide to accumulate at the inner membrane. The exclusion of the inner membrane is not as efficient as that of the outer membrane, which has now been permeabilized, hence the cell fluoresces red and appears to be "dead" even though the cell continues to maintain it's transmembrane gradients required for energy generation and ion selectivity. Care must obviously be taken during the intetation of fluorescent probes (using appropriate controls) to ensure that results are reliable.

Other research was performed using the gram positive isolate, *Lactococcus lactis* ATCC 11454, as a representative nisin-producing lactic acid-producing bacterium commonly found on meats and in meat processing plants. This particular nisin-producing strain of *L. lactis* is known to inhibit many gram positive enteric organisms, hence it was postulated that this organism may be employed for the community-level inhibition of detrimental bacteria. Agar plate inhibition studies using 10 $\mu$l drops of cell-free effluent filtrate from flow-cell cultured *L. lactis* revealed an ~25 mm zone of inhibition of spread-plated *Listeria monocytogenes*. Flow cell co-culture inhibition studies involving *L. lactis* and *L. monocytogenes* were then performed, allowing the *L. monocytogenes* to colonize the flow cell

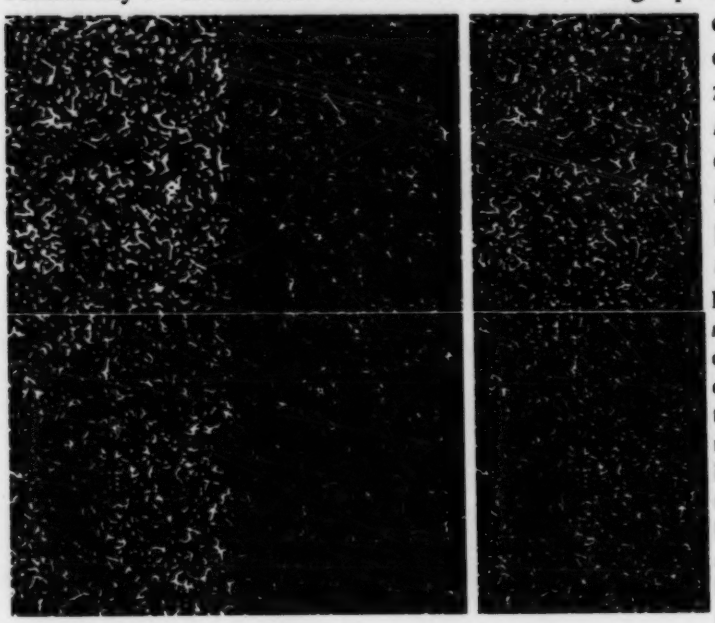


**Figure 1.** Two random CLSM images of *L. monocytogenes* biofilm bacteria grown in flow cells to which *L. lactis* ATCC 11454 (a nisin-producing strain) cells were added. Biofilms were then stained with the BacLight™ Live/Dead fluorescent viability probe. To the left of each merged image is the dual-channel image showing fluorescence from the green (left) and red (right) wavelengths. The left hand image shows the living *L. lactis* cells (green pseudocolor), whereas the right hand image shows the dead *L. monocytogenes* cells (red pseudocolor).

surface prior to addition of the *L. lactis* nisin-producing strain. **Figure 1** shows results of these co-culture studies where the previously established *L. monocytogenes* biofilm cells were apparently killed after the introduction of *L. lactis* ATCC 11454. The predictable and directed antagonism of certain beneficial biofilm bacteria are currently being examined for the control of food borne pathogens.

***Study area 4. Monitoring target microorganisms within complex microbial communities.***

**Project objective.**

A secondary element of this research was to investigate the potential for protective microbial communities to prevent pathogens from invading foods. In order to monitor the survival and proliferation of target pathogens within a complex microbial community, quantitative analytical methods needed to be developed. The methods developed were primarily based on conservative, non-toxic labeling of target cells via genetic transformation with the gene for the green fluorescent protein.

**Summary of results.**

In order to determine how pathogen organisms proliferate in complex systems, methods for identifying specific bacterial isolates were needed. While we have examined and developed protocols for the use of fluor tagged 16s rRNA-oligonucleotides as specific probes for monitoring different organisms, we have determined that viable, non-toxic and non-destructive approaches are preferrable. One such approach which has seen rapid development and acceptance is based on the genetic transformation of target organisms with plasmids containing the green fluorescent protein (GFP). The use of GFP as a stable, conservative fluorescent marker of food isolate bacteria permits the microscopic detection of bacteria in flow cell model systems, or the direct detection of GFP+ colonies on the surfaces of agar plates. Recent advances resulting from altering the spectral adsorption and emission properties of green fluorescent protein (GFP) have led to development of fade resistant fluors which are orders of magnitude more fluorescent than the wild-type gene. These can be excited by long-wave length UV light (~380nm) from a hand-held UV lamp for identification of GFP+ colonies on agar plates. A modified GFP (gfpmut3+) has previously been utilized during the current study to provide stable GFP+ strains of *Pseudomonas spp.*

Accordingly, bacterial transformations have been performed using the following *GFP* plasmid constructs: pGFP (a wild-type GFP), pEGFP (a red-shifted variant), and pGFPuv (a long-wavelength excitable variant). Bacteria transformed by these different GFP constructs vary in terms of their excitation and emission responses. For example, the pGFPuv was optimized for maximal fluorescence when excited by UV light (360-400 nm) as well as for enhanced expression of the *gfp* gene by recipient bacteria. The GFPuv variant is consequently well-suited for GFP expression and subsequent detection using a hand-held UV light. The pEGFP red-shifted variant contains mutations in the chromophore which shifts the maximal excitation peak to ~490 nm (the major line of excitation for argon ion based laser systems), resulting in more efficient chromophore excitation; it's fluorescence emission is ~35 fold more fluorescent than wild-type GFP as a consequence of a higher extinction coefficient (Em; 53,000 $cm^{-1}$ $M^{-1}$ at 489 nm). Consequently, bacteria expressing these genes are much easier to detect using microscopy.

The electroporation protocol for gram negative bacteria has worked quite well for *E. coli* and *Salmonella spp.*, with transformation efficiencies of approximately $10^5$-$10^6$. **Figure 1** shows an agarose gel of uncut plasmid DNA from transformed *E. coli* and *Salmonella spp.* It is apparent from this analysis, and more conclusively by visual observation of the GFP fluorescent phenotype of ampicillin-resistant transformants, that the various GFP plasmids were successfully introduced into various target bacteria strains. The list of organisms which have successfully been transformed via either electroporation or tri-parental matings are shown in **Table 1.** The gram positive meat bacteria have not

yet been successfully transformed. We have recently obtained an *E. coli* strain containing the pCM19 plasmid (Hansen et al., 2001) successfully used by others to transform gram positive isolates such as *Streptococcus* sp., *Lactococcus lactis*, and also *Listeria* sp. Attempts to transform *L. monocytogenes* 4 are currently in progress.

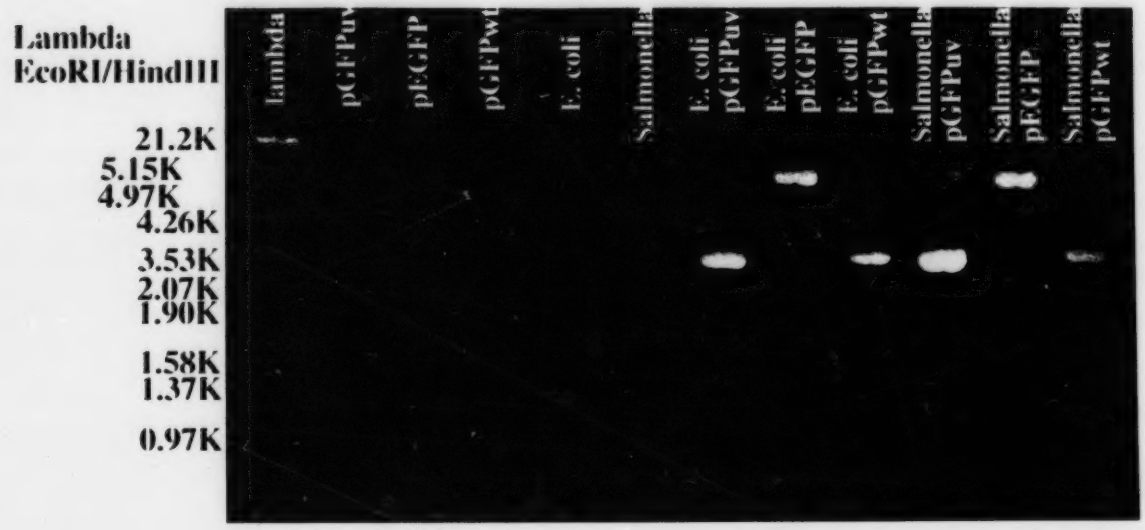



**Figure 1.** Agarose gel of uncut plasmid DNA from either: 1) pGFPuv, pEGFP, or pGFPwt plasmid DNA, 2) parent strains of *E. coli* strain 8 or *Salmonella sp.*, and 3 *E. coli* and *Salmonella sp.* transformed with pGFPuv, pEGFP, or pGFPwt plasmid DNA. Note the presence of a band of DNA corresponding with the 3.4/3.3 Kb plasmid(s), as well as a second band near the 15 Kb position. These secondary bands are circular plasmid DNA, as opposed to supercoiled plasmid DNA. Note the presence of similar bands in the transformed *E. coli* and Salmonella strain. It is interesting that while all *Salmonella* and *Escherichia* strains exhibited the GFP phenotype, the pEGFP gel lacked the 3.3Kb band on the gels. While the reason for this is not clear, it is apparent from both acquired ampicillin resistance as well as acquired GFP fluorescence that this strain was successfully transformed by pEGFP.

Examples of GFP-transformed *E. coli* 8 as viewed using microscopy (with UV excitation) or on agar plates (with long-wave UV light from a hand held lamp) are shown in Figures 2 and 3.

Table 1. List of bacterial isolates transformed with either pGFPuv, pEGFP, pGFPwt, pGFPmut3, or Tn5-gfp*.

| Strain | GFPuv | EGFP | GFPwt | GFPmut3 |
|---|---|---|---|---|
| ***Pseudomonas fluorescens D23*** | - | - | - | + |
| ***Escherichia coli 8*** | + | + | + | - |
| ***Salmonella sp.*** | + | + | + | - |
| ***Pseudomonas fluorescens BD1*** | - | - | - | + |
| ***Pseudomonas fluorescens BS2*** | - | - | - | + |
| ***Pseudomonas fluorescens CC840406-E*** | - | + | - | + |
| ***Salmonella* Enteritidis ATCC 4931*** | - | + | - | - |

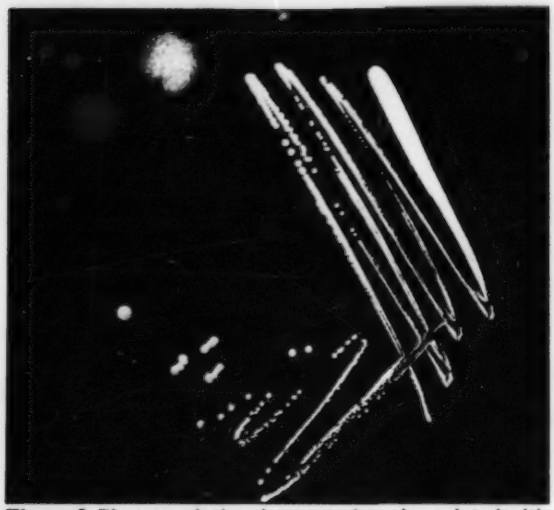


**Figure 2.** Photograph showing agar plates inoculated with *E. coli* 8 transformed with either pGFPuv, pEGFP, or pGFPwt and illuminated with long-wave UV light. Inset (arrow) shows the difference between the gfp-positive phenotype and the non-fluorescent parent *E. coli* strain as viewed under low-power epifluorescent microscopy.

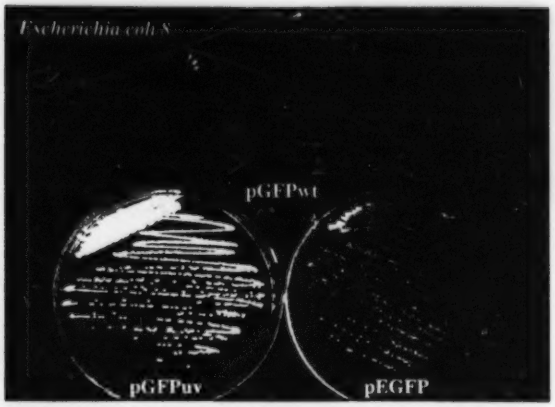



**Figure 3.** Photograph showing agar plates inoculated with *E. coli* 8 transformed with either pGFPuv, pEGFP, or pGFPwt and illuminated with long-wave UV light.

*GFP*-labeled organisms have subsequently been used to track the reproductive success of specific organisms grown in microbial communities cultivated in either flow cells or on the surfaces of meat disks. Early experimentation involving Tn5-*gfp* mutants of *E. coli* 8 or *S.* Enteritidis in biofilms have resulted in successful monitoring by SCLM for more than a week. An initial problem which hindered efficient monitoring of target organisms over extended periods was that the gfp fluorescence of organisms grown over time (in the presence of other competing microorganisms) was quite weak, and hence it was not clear whether we were able to quantify all of the target organisms. We subsequently evaluated different "resuscitation" strategies whereby we could increase the synthesis of *de novo* gfp protein; it was determined that the application of low carbon concentrations and higher flow rates (50 mg $L^{-1}$ TSB; 10 ml $h^{-1}$ flow velocity for 1 hr) resulted in excellent gfp fluorescence emission required for reliable monitoring of target cells in biofilms. **Figure 4** shows a number of meat isolate bacteria grown either in pure culture or in co-culture with other bacteria. In this presentation, *E. coli* and *S.* Enteritidis have been transformed using gfp and monitored using CLSM without the need for fluorescent staining. Note the tendency for *S. enteritidis* cells to become surrounded by *P. fragi* cells when the two bacteria were introduced into the flow cell at the same time. When *P. fragi* cells were allowed to colonize the flow cells prior to introduction of *S. enteritidis* cells, the two strains were not observed to aggregate in this manner.

GFP-labeled *S. enteritidis* were also examined using differential fluorescence of colonies on agar plates. **Figure 5** shows the relative number of *S. enteritidis* cells (the gfp-labeled target organism) and meat microflora community (isolated from the blood and serum in vacuum-packaged fresh, red meat aged for 14 days) bacteria when grown alone or in combination. Note the ~90% reduction in the number of *S. enteritidis* cells when grown in the presence of the community.

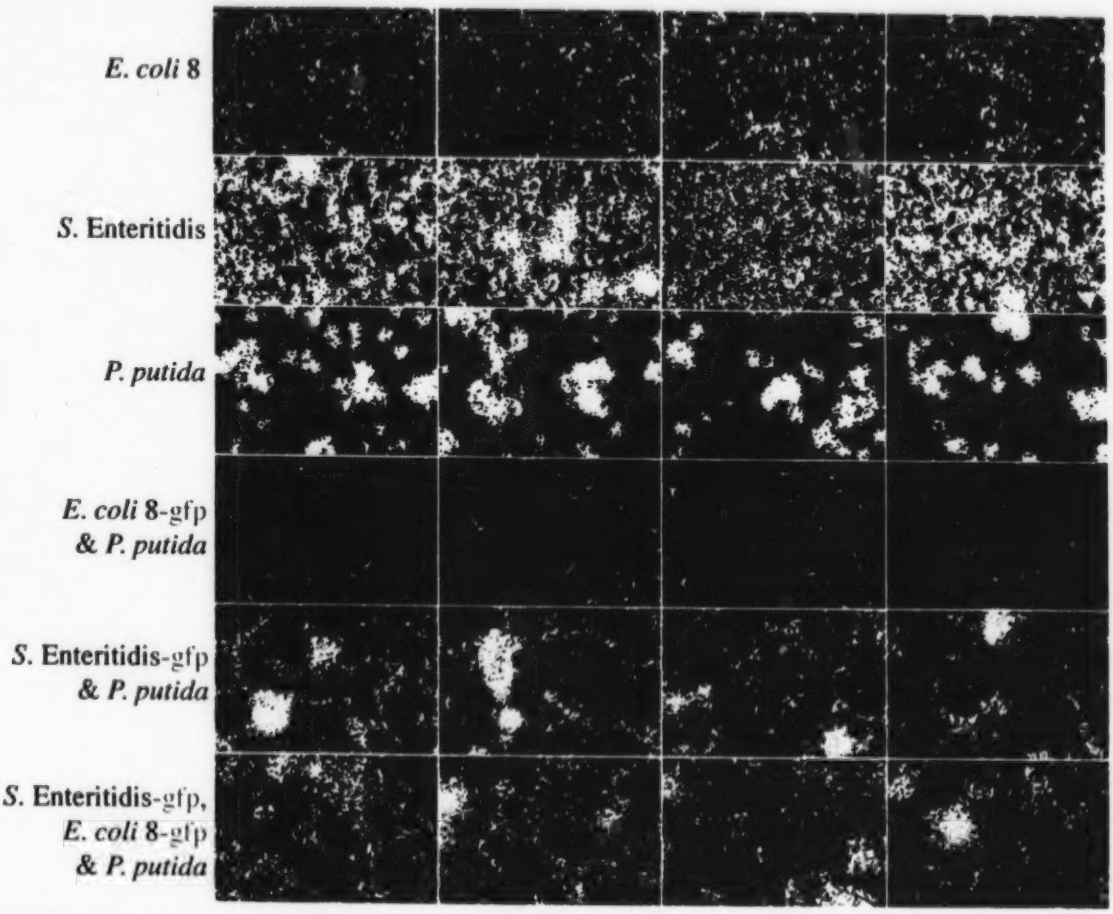
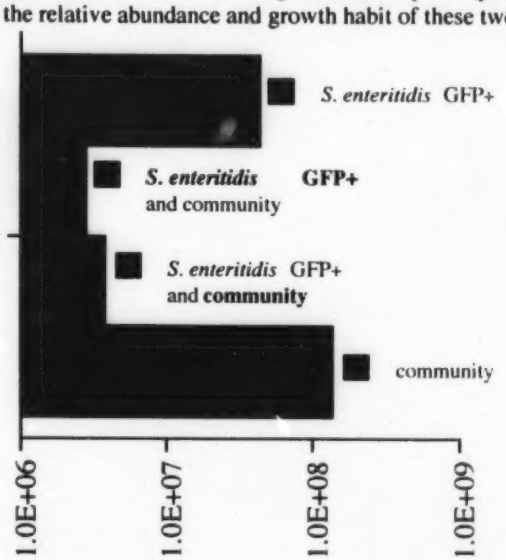



**Figure 4.** Top three rows show CLSM images of 48 h pure culture biofilms of *E. coli* 8, *S.* Enteritidis, and *P. putida* at 4 random locations stained with STYO 9. Bottom three rows show CLSM dual channel images of the above organisms grown as co-culture mixtures (see the left of figure for organism combination key). In the bottom 3 rows, one or more co-cultured organisms were conservatively labeled with gfp; these organisms fluoresced in the green wavelengths (pseudocolored green). The other bacteria were stained with a red-fluorescence emitting nucleic acid probe (pseudocolored red). Merging of these two images provides detail as to the relative abundance and growth habit of these two isolates.

**Figure 5.** Viable cells present on meat discs following cultivation at room temperature on 10% TSB agar for 96 h with either *S. enteritidis* (GFP+), *S.* Enteritidis GFP and a meat microflora inocula, or the meat microflora inoculum alone. Graphs shows the log bacterial counts obtained per $cm^2$ meat disc surface. Note that the bold-faced text indicates whether the counts indicate the number of *S.* Enteritidis cells (determined by counting GFP+ colonies) or the number of other community microflora organisms (counts of non-GFP colonies).



*Overall Conclusions*

1) While PDP enhanced the efficacy of various compounds (most notably, TSP) in model systems, the complex chemistry nature of meats, especially the buffering tendency of meat sera, interfered with a similar outcome on meat surfaces. While different agents have been tested (i.e., organic acids, nisin, and myristyl-L-methionine) we continue to explore whether this approach may yet yield positive results on either meat surfaces, or on meat processing associated materials. However, our findings indicate that the antimicrobial agent chosen must not interact with either the plasmolyzing agent, nor be dependent upon pH, for efficacy enhanced antimicrobial effect.

2) Due to the fact that TSP is an antimicrobial agent which currently sees application in poultry and red meat processing in North America, care must be taken to avoid sensitizing organisms such as *S.* Enteritidis to other antimicrobial treatments which might be encountered after TSP treatment. Accordingly, any cells which are not killed by TSP might ultimately become more resistant to other antimicrobial agents. Our results indicated that cells pretreated with sublethal TSP became more resistant to otherwise lethal alkaline stress factors, as well as heat treatments. This phenomenon is known as cross-protection

3) It is hoped that once the molecular basis for acquired TSP resistance is delineated, engineered strategies may be formulated whereby cross-protection may be eliminated or minimized.

4) Direct methods for monitoring target organisms, such as foodborne pathogens, based on GFP-tagged organisms is quite promising. Such approaches are anticipated to yield insights to the complex, interactive ecology that typifies meats and meat processing environments and also supply essential data on how these organisms can be controlled and/or inhibited via either antimicrobial treatments, or antagonistic microflora.

**Project Photos**
Glossy photos or graphics contained in this report are available on request.

**Acknowledgements**
The Agriculture Development Fund has been acknowledged for financial support at all meetings and on all publications where this material was, or will be, presented. We thank ADF for their generous support in our research endeavors.

**Project personnel**
The following personnel played significant roles in this research.
Dr. Darren Korber - Research Associate and PI
Dr. Gideon Wolfaardt - Research Associate
Bala Sampathkumar - Ph.D. candidate
Brij Verma - Technician
Eleonora Bellissimi - Summer student
Karthikeyan Subramanian - Technician

**Project Equipment**
nil

***Project Developed Materials.***


Sampathkumar, B., Khachatourians, G.G. and D.R. Korber. 2002. Trisodium phosphate permeabilizes *Salmonella Enteritidis* by disrupting the outer membrane. Appl. Environ. Microbiol. In submission.

Korber, D.R., Greer, G.G., Wolfaardt, G.M. and S. Kohlman. 2002. Efficacy enhancement of trisodium phosphate against spoilage and pathogenic bacteria in model biofilms and on adipose tissue. J. Food Prot. In press.

Karthikeyan, S., Korber, D.R., Wolfaardt, G.M., and Caldwell, D.E. 2001. Adaptation of bacterial communities to environmental transitions from labile to refractory substrates. Internatl. Microbiol. 4:73-80.

Korber, D.R. 2001. Image analysis of microorganisms. American Society for Microbiology Workshop, May 19-20, Orlando, FL.

Sampathkumar, B., Khachatourians, G.G., Wolfaardt, G.M., and Korber, D.R. 2001. Trisodium Phosphate permeabilizes *Salmonella enteritidis* by disrupting the outer membrane. Abstract. May 18-24. ASM Annual Meeting, Orlando, FL.

Saftic, S., Wolfaardt, G.M., and D.R. Korber, 2000. What do microbes see - the need to standardize surfaces and surface treatment. ASM Biofilms 2000, July 10-14, Big Sky, Mt.

Sampathkumar, B., Khachatourians, G.G., Wolfaardt, G.M., and Korber, D.R. 2000. Trisodium phosphate treatment induces thermotolerance in *Salmonella enteritidis*. ASM Annual Meeting, Anaheim Ca. Abstract.

Karthikeyan, S., Korber, D.R., Wolfaardt, G.M., and Caldwell, D.E. 2000. Monitoring the Organization of Microbial Biofilms. In: Handbook of Bacterial Adhesion. (An, H. Ed.) Humana Press, Totowa, N.J., pp. 171-188.

Korber, D.R. 2000. Determination of antimicrobial agent efficacy in model and meat systems. Institute of Food Technologists Annual Meeting, Dallas, Tx. Abstract.

Saftic, S. Bressel, A., Wolfaardt, G.M., Korber, D.R. and Schubert, J. 2000. Methods to characterize and monitor biofilms in distribution systems. South African Society of Microbiology Annual Meeting. Published abstract.

Karthikeyan, S., Wolfaardt, G.M., Korber, D.R. and D.E. Caldwell. 1999. Identification of synergistic interactions among organisms in biofilms by digital image analysis. Internatl. Microbiol. 2: 241-250.

Korber, D.R., Wolfaardt, G.M. and Greer, G.G. 1998. Enhanced antimicrobial efficacy against meat spoilage bacteria. Abstract CSM Annual Meeting, Guelph, Ont.

Korber, D.R. and G.M. Wolfaardt, 1998. Efficacy enhancement of antimicrobial agents for control of attached bacteria. Western Group Meeting on Meat Safety and Preservation. March 6, Saskatoon, Sask.

Karthikeyan, S., Wolfaardt, G.M., Korber, D.R., and Caldwell, D.E. 1998. Effect of a substrate shift on spatial organization within a biofilm community. Abstract. International Symposium on Microbial Ecology (ISME-8), Halifax, N.S.

Wood, P., Jones, M., Korber, D., Wolfaardt, G., and Gilbert, P. 1997. Surface catalyzed hygiene, *In:* Biofilms: Community Interactions and Control (J. Wimpenny, P. Handley, P. Gilbert, H. Lappin-Scott, and M. Jones, Eds.). Bioline, Cardiff. UK., pp. 227-234.


Two other manuscripts, 3 abstracts (B. Sampathkumar et al.) and one review article (Korber) are in various stages of preparation or completion; all will acknowledge ADF for their contributions to this research.

**Expense Statement**
Prepared by the Department of Financial Services.

***Key References***


Anderl, J.N., M.J. Franklin, and P.S. Stewart. 2000. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. Antimicrob. Agents Chemother. 44:1818-1824.

Arnold, J.W. and S. Silvers. 2000. Comparison of poultry processing equipment surfaces for susceptibility to bacterial attachment and biofilm formation. Poultry Sci. 79:1215-1221.

Auty, M.A.E., G.E. Gardiner, S.J. McBrearty, E.O. O'Sullivan, D.M. Mulvihill, J.K. Collins, G.F. Fitzgerald, C. Stanton, and R.P. Ross. 2000. Direct *in situ* viability assessment of bacteria in probiotic dairy products using viability staining in conjunction with confocal scanning laser microscopy. Appl. Environ. Microbiol. 67:420-425.

Bagge, D., M. Hjelm, C. Johansen, I. Huber, and L. Gram. 2001. *Shewanella putrefaciens* adhesion and biofilm formation on food processing surfaces. Appl. Environ. Microbiol. 67:2319-2325.

Bender, F. G., and E. Brotsky. 1992. Process for treating poultry carcasses to control salmonella growth. U.S. Patent No. 5,143,739.

Berry, E.D. and C.N. Cutter. 2000. Effects of acid adaptation of *Escherichia coli* O157:H7 on efficacy of acetic acid spray washes to decontaminate beef carcass tissue. Appl. Environ. Microbiol. 66:1493-1498.

Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Brown, M.R.W., D.G. Allison, and G. Gilbert. 1988. Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? J. Antimicrob. Chemother. 22:777-780.

Cabedo, L., J.N. Sofos, G.R. Schmidt, and G.C. Smith. 1997. Attachment of *Escherichia coli* O157:H7 and other bacterial cells grown in two media to beef adipose and muscle tissues. J. Food Prot. 60:102-106.

Capita, R., C. Alonso-Calleja, M.C. Garcia-Fernádez, and B. Moreno. 2001. Influence of strain and trisodium phosphate concentration on growth parameters of *Listeria monocytogenes in vitro*. Lett. Appl. Microbiol. 32:428-432.

Capita, R., C. C. Alonso, M. Sierra, B. Moreno, G. F. M. del-Camino. 2000. Effect of trisodium phosphate washing on the sensory evaluation of poultry meat. Meat Sci. 55:471-474.

Carneiro de Melo, A.M.S., C.A. Cassar, and R.J. Miles. 1998. Trisodium phosphate increases sensitivity of gram-negative bacteria to lysozyme and nisin. J. Food Prot. 61:839-844.

Centers for Disease Control. 1993. Update; multistate outbreak of *Escherichia coli* O157:H7 infections from hamburgers- Western United States, 1992-1993. Morbid. Mortal. Weekly Rep. 42:258-263.

Centers for Disease Control. 2000. Preliminary FoodNet Data on the Incidence of Foodborne Illnesses - Selected Sites, United States, 1999. Morbid. Mortal. Weekly Rep. 49:201-205.

Coppen, P., S. Fenner, G. Salvant. 1998. Antimicrobial efficacy of AvGard carcass wash under industrial processing conditions. British Poultry Sci. 39:229-234.

Csonka, J. S. 1991. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. 53:121-147.

Cutter, C.N. and G.R. Siragusa. 1995. Population reductions of gram–negative pathogens following treatments with nisin and chelators under various conditions. J. Food Prot. 58:977-983.

Cutter, C.N. and M. Rivera-Betancourt. 2000. Interventions for the reduction of *Salmonella typhimurium*

DT 104 and non–O157:H7 enterohemorrhagic *Escherichia coli* on beef surfaces. J. Food Prot. 63:1326-1332.

Delaquis, P.J., C. Gariepy, and D. Montpetit. 1992. Confocal laser scanning microscopy of porcine muscle colonization by meat spoilage bacteria. Food Microbiol. 9: 147-153.

Delmore, R. J., J. N. Sofos, G. R. Schmidt, K. E. Belk, W. R. Lloyd, and G. C. Smith. 2000. Interventions to reduce microbiological contamination of beef meats. J. of Food Prot. 63:44-50.

Desai, M., T. Buhler, P.H. Weller, and M.R.W. Brown. 1998. Increasing resistance of planktonic and biofilm cultures of *Burkholderia cepacia* to ciprofloxacin and ceftazidime during exponential growth. J. Antimicrob. Chemother. 42:153-160.

Dickson, J. S., and M. E. Anderson. 1992. Microbiological decontamination of food animal carcasses by washing and sanitizing systems: a review. J. Food Prot. 55:133-140.

Dickson, J.S. 1990. Surface moisture and osmotic stress as factors that affect the sanitizing of beef tissue surfaces. J. Food Prot. 53:674-679.

Dickson, J.S. 1991. Attachment of *Salmonella typhimurium* and *Listeria monocytogenes* to beef tissue: effects of inoculum level, growth temperature and bacterial culture age. Food Microbiol. 8:143-151.

Dickson, J.S., C. N. Cutter, and G.R. Siragusa. 1994. Antimicrobial effects of trisodium phosphate against bacteria attached to beef tissue. J. Food Prot. 57:952-955.

Dorsa, W.J., C.N. Cutter, and G.R. Siragusa. 1997. Effects of acetic acid, lactic acid and trisodium phosphate on the microfloras of refrigerated beef carcass tissue inoculated with *Escherichia coli* O157:H7, *Listeria* innocua and *Clostridium sporogenes*. J. Food Prot. 60:619-624.

Dorsa, W.J., C.N. Cutter, and G.R. Siragusa. 1998. Long-term effect of alkaline, organic acid or hot water washes on the microbial profile of refrigerated beef contaminated with bacterial pathogens after washing. J. Food Prot. 61:300-306.

Dykes, G. A. 1999. Physical and metabolic causes of sub-lethal damage in *Listeria monocytogenes* after long-term chilled storage at 4°C. J. Appl. Microbiol. 87:915-922.

El-Kest, S. E., and E. H. Marth. 1992. Transmission electron microscopy of unfrozen and frozen/thawed cells of *Listeria monocytogenes* treated with lipase and lysozyme. J Food Prot. 55:687-696.

Farkas, J. 1998. Irradiation as a method for decontaminating food: A review. Int. J. Food Microbiol. 44:189-204.

Federal Register. 1982. Meat and poultry products: phosphates and sodium hydroxide. Fed. Regist. 47:10779.

Federal Register. 1994. Use of trisodium phosphate on raw, chilled poultry carcasses. Fed. Regist. 59:551-554.

Flahaut, S., A. Hartke, J. C. Giard, and Y. Auffray. 1997. Alkaline stress response in *Enterococcus faecalis*: adaptation, cross-protection, and changes in protein synthesis. Appl. Environ. Microbiology. 63:812-814.

Gibson, H., J.H. Taylor, K.E. Hall, and J.T. Holbak. 1999. Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. J. Appl. Microbiol. 87:41-48.

Giesse, J. 1992. Experimental process reduces salmonella on poultry. Food Technol. 46:112.

Gill, C.O. 1998. Microbiological contamination of meat during slaughter and butchering cattle, sheep and pigs, p. 188-157. *In* A.R. Davies and R.G. Board (eds.), The microbiology of meat and poultry, Blackie Academic, London.

Greer, G.G. and B.D. Dilts. 1995. Lactic acid inhibition of cold tolerant spoilage bacteria and pathogens on pork. Int. J. Food Microbiol. 25:141-151.

Greer, G.G., A. Paquet, and B.D. Dilts. 2000. Inhibition of *Brochothrix thermosphacta* in broths and pork by myristoyl-L-methionine. Food Microbiol. 17:177-183.

Hanson, M.C., Palmer, Jr., R.J., Udsen, C., White, D.C., and S. Molin. 2001. Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. Microbiology 147: 1383-1391.

Helander, I. M., P. H. Mäkelä, O. Westphal, and E. T. Rietschel. 1996. Lipopolysaccharides, p. 462-471. In R. A. Meyers (ed.), Encyclopaedia of molecular biology and molecular medicine, Vol. 3. VCH, Weinheim, Germay.

Helander, I., and M. Vaara. 1987. Reversible binding of *Salmonella typhimurium* lipopolysaccharides by immobilized protamine. Eur. J. Biochem. 163:51-55.

Hollender, R., F. G. Bender, R. K. Jenkins, and C. L. Black. 1993. Research note: Consumer evaluation of chicken treated with a trisodium phosphate application during processing. Poultry Sci. 72:755-759.

Hood, S.K. and E.A. Zottola. 1997. Isolation and identification of adherent gram-negative microorganisms from four meat-processing facilities. J. Food Prot. 60:1135-1138.

Hwang, C., and L. R. Beuchat. 1995. Efficacy of selected chemicals for killing pathogenic and spoilage microorganisms on chicken skin. J. Food Prot. 58:19-23.

Kim, C. R., and D. L. Marshall. 1999. Microbiological, colour and sensory changes of refrigerated chicken legs treated with selected phosphates. Food Res. Int. 32:209-215.

Kim, J., and M. F. Slavik. 1994a. Trisodium phosphate treatment of beef surfaces to reduce *Escherichia coli* O157:H7 and *Salmonella typhimurium*. J. Food Sci. 59:20-22.

Kim, J., and M. F. Slavik. 1994b. Removal of *Salmonella typhimurium* attached to chicken skin by rinsing with trisodium phosphate solution: scanning electron microscopic examination. J. Food Safety. 14:77-84.

Kim, J., M. F. Slavik, M. D. Pharr, D. P. Raben, C. M. Lobsinger, and S. Tsai. 1994. Reduction of *Salmonella* on post-chill chicken carcasses by trisodium phosphate treatment. J. Food Safety 14:9-17.

Korber, D.R., A. Choi, G.M. Wolfaardt, and D.E. Caldwell. 1996. Bacterial plasmolysis as a physical indicator of viability. Appl. Environ. Microbiol. 62:3939-3947.

Korber, D.R., A. Choi, G.M. Wolfaardt, S.C. Ingham, and D.E. Caldwell. 1997. Substratum topography influences susceptibility of *Salmonella enteritidis* biofilms to trisodium phosphate. Appl. Environ. Microbiol. 63:3352-3358.

Korber, D.R., G.A. James, and J.W. Costerton. 1994. Evaluation of fleroxacin activity against established *Pseudomonas fluorescens* biofilms. Appl. Environ. Microbiol. 60:1663-1669.

Korber, D.R., J.R. Lawrence, B. Sutton, and D.E. Caldwell. 1989. Effect of laminar flow velocity on the kinetics of surface recolonization by mot$^+$ and mot$^-$ *Pseudomonas fluorescens*. Microb. Ecol. 18:1-19.

Kumar, C.G. and S.K. Anand. 1998. Significance of microbial biofilms in food industry: A review. Int. J. Food Microbiol. 42:9-27.

Lawrence, J.R., D.R. Korber, G.M. Wolfaardt, and D.E. Caldwell. 1997. Analytical imaging, p. 29-51. *In* C.J. Hurst, G.R. Knudsen, M.J. McInerney, L.D. Stetzenback, and M.V. Walter (eds.), Manual of environmental microbiology, ASM Press, Washington, D.C.

Lee, R. M., P. A. Hartman, D. G. Olson, and F. D. Williams. 1994. Metal ions reverse the inhibitory effects of selected food-grade phosphates in *Staphylococcus aureus*. J. Food Prot. 57:284-288.

Leive, L. 1974. The barrier function of gram-negative envelope. Ann. N. Y. Acad. Sci. 235:109-127.

Lou, Y. and A.E. Yousef. 1997. Adaptation to sublethal environmental stresses protects *Listeria monocytogenes* against lethal preservation factors. Appl. Environ. Microbiol. 63:1252-1255.

Lugtenberg, B., and L. van Alpen, 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochim. Biophys. Acta 737:51-

115.

Mackey, B. M., K. Forestière, N. S. Isaacs, R. Stenning, and B. Brooker. 1994. The effect of high hydrostatic pressure on *Salmonella thompson* and *Listeria monocytogenes* examined by electron microscopy. Lett. Appl. Microbiol. 19:429-432.

Mager, J., M. Kuczynski, G. Schatzberg, and Y. Avi-Dor. 1956. Turbidity changes in bacterial suspensions in relation to osmotic pressure. J. Gen. Microbiol. 14:69-75.

Marriott, N.G., A.M. Ahmed, and H.A. Abl El-Rhman. 1996. The effect of phosphates on the proliferation of *Listeria monocytogenes*. Meat Focus Internat. 5:466-468.

Mattick, K.L., F. Jørgensen, J.D. Legan, M.B. Cole, J. Porter, H.M. Lappin-Scott, and T.J. Humphrey. 2000. Survival and filamentation of *Salmonella enterica* serovar *enteritidis* PT4 and *Salmonella enterica* serovar *typhimurium* DT104 at low water activity. Appl. Environ. Microbiol. 66:1274-1279.

Mendonca, A. F., T. L. Amoroso, and S. J. Knabel. 1994. Destruction of gram-negative food-borne pathogens by high pH involves disruption of the cytoplasmic membrane. 60:4009-4014.

Michiels, C.W., M. Schellekens, C.C.F. Soontjens, and K.J.A. Hauben. 1997. Molecular and metabolic typing of resident and transient fluorescent pseudomonad flora from a meat mincer. J. Food Prot. 60:1515-1519.

Mittelman, M.W. 1998. Structure and functional characteristics of bacterial biofilms in fluid processing operations. J. Dairy Sci. 81:2760-2764.

Morris, C.A., L.M. Lucia, J.W. Savell, and G.R. Acuff. 1997. Trisodium phosphate treatment of pork carcasses. J. Food Sci. 62:402-405.

Mullerat, J., B.W. Sheldon, and N.A. Klapes. 1995. Inactivation of Salmonella species and other food-borne pathogens with Salmide®, a sodium chlorite-based oxyhalogen disinfectant. J. Food Prot. 48:535-540.

Murray, R. G. E. 1957. Can J. Microbiol. 3:531-532.

Murray, R. G. E., P. Steed, and H. E. Elson. 1965. The location of the mucopeptide in sections of the cell wall of *Escherichia coli* and other gram-negative bacteria. Can. J. Microbiol. 11:547-560.

Nikaido, H., and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. 49:1-32.

Norwood, D.E. and A. Gilmour. 2000. The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm. J. Appl. Microbiol. 88:512-520.

Palumbo, S.A., J.E. Call, P.H. Cooke, and A.C. Williams. 1995. Effect of polyphosphates and NaCl on *Aeromonas hydrophila* K144. J. Food Safety 15:77-87.

Podolak, R. K., J. F. Zayas, C. L. Kastner, and D. Y. C. Fung. 1996. Inhibition of *Listeria monocytogenes* and *Escherichia coli* O157:H7 on beef by application of organic acids. J. Food Prot. 59:370-373.

Rhône-Poulenc. 1992. AvGard™ TSP Salmonella reduction process. Rhône-Poulenc, Princeton. NJ.

Ronner, A.B. and A.C.L. Wong. 1993. Biofilm development and sanitizer inactivation of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and Buna-n rubber. J. Food Prot. 56:750-758.

Salvant, G. P. Coppen, J. C. Allo, M. J. Laisney, M. T. Toquin, F. Humbert, P. Colin. 1997. Effects of AvGard treatment on the microbiological flora of poultry carcases. 38:489-498.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Schagger, H. and G. V. Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide electrophoresis for the separation of proteins in the range from 1 to 100 KDa. Anal. Biochem. 166:368-379.

Scudamore, R. A., T. J. Beveridge, and M. Goldner. 1979. Outer-membrane penetration barriers as

components of intrinsic resistance to beta-lactam and other antibiotics in *Escherichia coli* K-12. Antimicrob. Agents Chemother. 15:182-189.

Slavik, M. F., J. Kim, M. D. Pharr, D. P. Raben, S. Tsai, and C. M. Lobsinger. 1994. Effect of trisodium phosphate on *Campylobacter* attached to post-chill chicken carcasses. J. Food Prot. 57:324-326.

Smulders, F. J. M., and G. G. Greer. 1998. Integrating microbial decontamination with organic acids in HACCP programmes for muscle foods: prospects and controversies. Int. J. Food Microbiol. 44:149-169.

Somers E.B., J.L. Schoeni, and A.C.L. Wong. 1994. Effect of trisodium phosphate on biofilm and planktonic cells of *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella typhimurium*. Int. J. Food Microbiol. 22:269-276.

Stevens, K. A., N. A. Klapes, B. W. Sheldon, and T. R. Klaenhammer. 1992. Effect of treatment conditions on nisin inactivation of gram-negative bacteria. J. Food Prot. 55:763-766.

Stewart, P.S., L. Grab, and J.A. Diemer. 1998. Analysis of biocide transport limitation in an artificial biofilm systems. J. Appl. Microbiol. 85:495-500.

Strange, R. E. 1964. Effect of magnesium on permeability control in chilled bacteria. Nature 203:1304-1305.

Taormina, P. J., and R. L. Beuchat. 1999. Comparison of chemical treatments to eliminate enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa seeds. J. Food Prot. 62:318-324.

Ulitzur, S. 1972. Rapid determination of DNA base composition by ultraviolet spectroscopy. Biochim. Biophys. Acta 272:1-11.

USDA 1996. United States Department of Agriculture Food Safety and Inspection Service. Federal Register, Vol. 61. No. 144.

Velázquez, L.C., M.E. Escudero, A.M.S. de Guzman. 2001. Antibacterial effects of different food-related phosphates using *Aeromonas hydrophila*. J. Food Prot. 64:195-200.

Wolfaardt, G.M., J.R. Lawrence, R.D. Robarts, and D.E. Caldwell. 1994. Multicellular organization in a degradative biofilm community. Appl. Environ. Microbiol., 60: 434-446.

Wong, A.C.L. 1998. Biofilms in food processing environments. J. Dairy Sci. 81:2765-2770.

Xiong, H., Y. Li, M. F. Slavik, and J. T. Walker. 1998a. Spraying chicken skin with selected chemicals to reduce attached *Salmonella typhimurium*. J. Food Prot. 61:272-275.

Xiong, H., Y. Li, M. F. Slavik, and J. T. Walker. 1998b. Chemical spray conditions for reducing bacteria on chicken skins. J. Food Sci. 63:699-701.

Yang, Z., Y. Li, and M. F. Slavik. 1998. Use of antimicrobial spray applied with an inside-outside birdwasher to reduce bacterial contamination on prechilled chicken carcasses. J. Food Prot. 61:829-832.

Zhuang, R. Y., and L. R. Beuchat. 1996. Effectiveness of Trisodium phosphate for killing *Salmonella Montevideo* on tomatoes. Lett. Appl. Microbiol. 22:97-100.

Zottola, E.A. and K.C. Sasahara. 1994. Microbial biofilms in the food processing industry – should they be a concern? Int. J. Food Microbiol. 23:125-148.